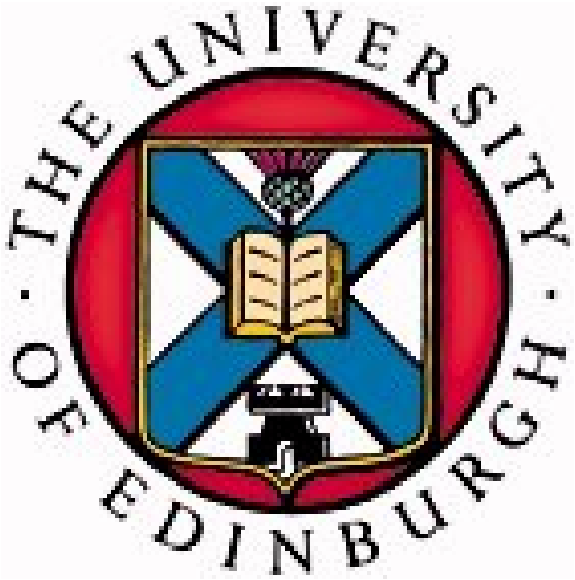

The Nutritional Control of Parasitism

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A thesis submitted towards the degree of Doctor of Philosophy at the University of Edinburgh
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Dedication

This work is dedicated to my Granny for her continuos love, support and belief in me.

Declaration

I hereby declare that this thesis is of my own composition and all assistance has been fully acknowledged. The results presented herein have not previously been submitted for any other degree or qualification.

Heidi Normanton

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Abstract

Expression of acquired immunity to gastrointestinal parasites usually breaks down during the periparturient period, which is characterised by an increased worm burden and nematode egg excretion. It is believed that this breakdown of immunity may have a nutritional basis, and that by reducing nutrient scarcity the lactating animal will be able to reduce her worm burden. Therefore, the aim of this thesis was to carry out four experiments to investigate the potential use of metabolisable protein as an alternative way to control gastrointestinal parasitism in periparturient animals. A lactating rat model was used to address this issue as lactating rats exhibit a breakdown of immunity to the gastrointestinal nematode *Nippostrongylus brasiliensis*. The first experiment (chapter two) aimed to verify that a reduction in worm burden is indeed related to changes in nutrient supply and not associated with changes in the gut environment. This was achieved by manipulating nutrient (litter) demand whilst nutrient supply was maintained constant. The results showed that the periparturient breakdown of immunity to *N. brasiliensis* (measured by a reduced worm burden) was sensitive to changes in nutrient demand and that these effects were independent of changes in the gut environment. The second experiment (Chapter Three) tested the effect of increased protein supply or reduced protein demand on the resistance to parasites in lactating rats whilst energy intake was kept constant. Under these conditions effects of protein supply could not be confounded with effects of any nutrient or energy intake. The results supported the view that under a restricted feeding regime, breakdown of immunity to *N. brasiliensis* (measured by a reduced number of eggs in the colon content) was sensitive to changes in protein scarcity. Following on from this, the next experiment (Chapter Four) assessed the effects of a gradual increase in protein supply on resistance and immune responses to *N. brasiliensis* in lactating rats. It was shown that as protein contents of the diets progressively increased, the number of worms and eggs present in the colon decreased. Although we found that differences in protein supply affected parasite burden, we found no affects of protein supply on local immune responses. This may have been due to the single sampling point used. Therefore, the objective of the last experiment (Chapter Five) was to assess temporal effects of increased protein supply on resistance and immune responses to *N. brasiliensis*. In agreement with previous experiments, the results showed that an increase in protein at times of protein scarcity improved resistance to *N. brasiliensis*, illustrated by a lower number of nematode eggs in the colon. The results also showed that local immune responses such as immunoglobulin levels (IgA, IgE & IgG_{2a}), RMCP II levels and goblet cell counts were affected by differences in protein supply at various time points post secondary infection. The potential application of using a lactating rat as a suitable model to fully understand the underlying immunological basis of relaxation in immunity during the periparturient period and its sensitivity to nutrient scarcity is considered in the General Discussion (Chapter Six).

Publications

Refereed Papers

H Normanton, J Houdijk, D Knox, I Kyriazakis (2007). The effects of changes in nutritional demand on gastrointestinal parasitism in lactating rats. *British Journal of Nutrition* (97), 104-110.

Conferences

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Chapter One

General Introduction

1.1 Introduction

Gastrointestinal parasitism is the major challenge to the health, welfare and production of foraging animals, including farm animals. For example, gastrointestinal nematode parasitism can have major detrimental effects on the efficiency of production of grazing animals, with production losses of up to 50% (Sykes 1994). In general, gastrointestinal parasitism reduces host performance. It can result in a reduced nutrient supply to the host through voluntary reductions in food intake (mainly for abomasal nematodes) and reduced food efficiency (mainly for small intestinal nematodes) (Houdijk & Athanasiadou, 2003). Until recently, gastrointestinal parasitism of farm animals has largely been controlled through the use of anthelmintics. However, due to the increasing prevalence of nematode resistance to drugs, and public concerns about chemical residues in the food chain and the environment (Houtert *et al.,* 1996), control of gastrointestinal nematodes with anthelmintics in farm animals is becoming progressively more difficult and unsustainable. Therefore, there has been a need to develop sustainable approaches to help control nematode infections in farm animals with less reliance on frequent chemotherapy intervention.

1.2 Alternative control of gastrointestinal nematodes

Alternative methods for the control of parasitism include breeding parasite resistant hosts by genetic selection (Gray, 1997), use of plants with anthelmintic properties (Hammond *et al*, 1997) and biological control of nematodes by using nematophagous

fungi (Thamsborg et al, 1999). Another such alternative is the enhancement of immunity to gastrointestinal nematodes through improved nutrition.

1.3. Nutrition and immunity

It is believed that improving nutrition can help control the degree of breakdown of immunity. During a secondary infection the expression of acquired immunity to parasites can break down. This breakdown in immunity has been observed in both growing and ageing animals. For example, type II ostertagiosis has been observed in growing calves at the end of their winter housing period. Inhibited larvae present within the gastrointestinal tract re-emerge which could be considered as a breakdown of immunity to gastrointestinal nematodes. It has been suggested that host nutrition, and the presence of body reserves in particular, may affect this breakdown of immunity (Berghen et al, 1990). Age-related breakdown of immunity is often associated with a reduced cellular immune response and delayed secondary immune responses (Chandra, 1989). However, it has been shown that supplementation and nutritional advice to ageing human subjects improve their immune responses in general, and their cellular immune responses in particular (Chandra, 1989). As well as during the periods of growth and ageing, host nutrition may affect the degree of breakdown of immunity during the periparturient period.

Therefore, during late pregnancy and lactation, animals often exhibit a degree of breakdown of immunity towards gastrointestinal parasites. This is often associated with an increased gastrointestinal nematode burden and excretion of nematode eggs.

This has an important epidemiological role, as the infected periparturient animal can be a major source of infection to her offspring, due to an increased worm burden which results in increased excretion of nematode eggs onto the pasture (Nieuwhof GF & Bishop SC, 2005).

In the past, the periparturient relaxation of immunity has been attributed to various factors such as seasonal effects, stress associated with parturition, lack of antigenic stimulation (Barger 1993) or the immunosuppressive role of certain hormones, such as prolactin, progesterone or corticosteriods (Coop *et al*, 1990). Although each of these factors probably influence the extent of the periparturient relaxation, the cause still remains unclear. More recently, a nutrient partitioning framework has been developed which suggests a possible nutritional basis for the relaxation in immunity (Coop & Kyriazakis, 1999).

1.4. A nutritional framework for the breakdown of immunity

The hypothesis that host nutrition can affect the degree of breakdown of immunity is based on the nutrient-partitioning framework of Coop & Kyriazakis (1999). It is suggested that an animal can ensure its survival and reproductive fitness by varying the degree of scarce nutrient resource (s) allocation to various body functions. A naive animal, i.e. without any prior experience of parasite infection, will be expected to prioritise acquisition of immunity over growth. This is because otherwise the animal may succumb to the adverse consequences of the parasitic burden before it reaches reproductive maturity (Coop & Kyriazakis, 1999). However, a re-infected

animal will have already built up an effective immune response, giving first priority to maintenance of body protein as this will guarantee the animal's survival in the short term. It is proposed that reproduction (pregnancy/lactation) is given the second highest priority as this ensures the survival of the animal in the long term. These priorities are not absolute but rather partial or graduated, which means that the degree of scarce nutrient allocation can vary for the different body functions at the same time. Expression of immunity, and then maintenance or growth of body reserves, are considered to have relatively low priorities for the allocation of scarce nutrients. Therefore, the proposed nutritional basis for the periparturient relaxation in immunity is that it occurs due to an increased nutrient requirement of the prioritised reproductive effort at times when nutrient supply is scarce (Coop & Kyriazakis, 1999).

The theory that improving the nutritional status of an animal reduces the production losses and mortality rates associated with parasitic nematode infections has been raised as far back as 1932, when it was suspected that improving host nutrition may affect gastrointestinal nematodes detrimentally (Clunies-Ross & Graham, 1932). More recently, studies using periparturient animals have shown that supplementing diets with fishmeal (Donaldson, 1998) or increasing the protein content of the diet resulted in reduced faecal egg counts and worm burdens (Houdijk *et al*, 2000, 2001). Therefore, there is an increasing body of evidence to support the view that host nutrition affects the degree of breakdown of immunity to gastrointestinal nematodes in periparturient animals (Chartier *et al*, 2000, Houdijk *et al*, 2000, 2001*a*,*b*), and

these results are consistent with the nutritional framework proposed by Coop & Kyriazakis (1999).

There have been other studies which have shown that the expression of immunity to gastrointestinal parasites is not sensitive to moderate changes in energy supply (Bown et al, 1991; Donaldson et al, 1998; Houdijk et al, 2000) but that other nutrients, such as vitamins and minerals (Koski & Scott, 2001) and n-3 fatty acid intake (Yaqoob, 2004) are associated with helping to reverse the breakdown of immunity. However, most research has concentrated on the effects of host protein nutrition on the degree of immunity breakdown. This seems reasonable since during lactation there is a dramatic increase in metabolisable protein (MP) requirement, and since voluntary protein intake may be insufficient to meet MP requirements during late pregnancy and lactation it is considered a scarce nutrient during the periparturient period (AFRC, 1993). In addition, many components of the effector arms of the immune system such as immunoglobulins and cellular products such as leukotrienes are proteinaceous in nature (Macrae, 1993; Coop & Holmes, 1996) and they would be expected to draw on MP resources (Coop & Kyriazakis, 1999).

It has been demonstrated using sheep, that factors that reduce MP pressure, such as decreased requirements for lactation associated with weaning, can terminate the periparturient rise of nematodes (O'Sullivan & Donald, 1970). In agreement with this study, other experiments have shown that parasitised single-rearing ewes have usually lower FEC and smaller worm burdens than their twin-rearing counterparts (Houdijk *et al* 2001; Donaldson *et al*. 1998; Kahn *et al*. 2003), whilst similar effects

have been seen in low-yielding dairy goats compared to high producing goats (Chartier *et al*. 2000). Again, more recently, it has also been shown that increasing scacre nutrient supply can help terminate the periparturient rise of nematodes. Donaldson (1998) conducted a study using twin-bearing ewes supplemented with the protein source fishmeal. The faecal egg count and worm burden of the ewes on a low level of fishmeal was significantly higher than the ewes which received a high level of fishmeal. Therefore, there is now an increasing body of evidence to support the view that host nutrition in general, and host protein nutrition in particular, affects the degree of breakdown of immunity to gastrointestinal nematodes in various species. The most recent research has investigated the effect of host protein nutrition on the degree of breakdown in immunity using a rodent model (Houdijk *et al*, 2003,2005).

1.5. Rodent model

The nutritional basis of breakdown of immunity to parasites has also been addressed in a rodent model using a rat and a common rodent nematode called *Nippostrongylus brasiliensis* (Fig 1). The rat was chosen as the experimental model as it is easy to manipulate the host's nutritional status due to the substantially elevated nutrient requirement during lactation (up to tenfold relative to maintenance (Pine *et al*, 1994), and its lactational response to changes in endogenous and dietary protein supply are well established (Jessop, 1997). In addition, rats acquire a relatively strong immunity to the small intestinal nematode *N. brasiliensis*, and immune rats rapidly expel adult *N. brasiliensis* during a secondary infection (Jarrett *et al*. 1968).

Figure 1 Adult Nippostrongylus brasiliensis

The most appropriate model to elucidate the nutritional basis of the breakdown of immunity to gastrointestinal nematodes would be one that exploits a secondary *N. brasiliensis* infection in the lactating rat. Although host lactation delays the expulsion of adult *N. brasiliensis* in a primary infection (Connan, 1970; Dineen & Kelly, 1972), these rats are not immune and therefore, the models are not appropriate to assess breakdown of immunity, as they do not represent a degree of a breakdown of a previously acquired immunity. In agreement with this view, Houdijk *et al* (2003) showed that a breakdown of immunity to *N. brasiliensis* occurs during re-infection in the lactating rat. Therefore, it was concluded that lactating rats could be used as a model to systematically elucidate a possible nutritional basis for the breakdown of immunity to parasites.

1.6. Life-cycle of *N. brasiliensis*

N. brasiliensis eggs hatch in the faeces from the host and develop from L_1 stage larvae to L_2 and then L_3 stage. In nature, the infective larvae (L3) then enter the host by penetrating the skin and enter the vasculatory system migrating to the lungs (Giacomin *et al*, 2005). In most experimental situations, the L_3 stage larvae are subcutaneously injected. Once inside the lungs, the larvae mature further to L_4 stage larvae and migrate via the trachea and oesophagus to the gastrointestinal tract. Having reached the small intestine, the larvae mature into adult egg producing worms. The larvae arrive in the lungs 18-48 hours later, where they carry on migrating towards the small intestine (Haley, 1962).

Our model uses a high-dose single infection, as this stimulates a more powerful and protective Th2 type immune response (Lawrence *et al*, 1996), which is an important aspect of the model when investigating the possible effects of increased MP supply on individual immune responses.

1.6.1. Primary infection

The establishment and expulsion of *N. brasiliensis* in the naive and immune rat are described as follows. During a primary infection, a proportion of the infective larvae dose is immobilised or destroyed before or during the course of migration via the

lungs to the intestine (**loss phase 1**). The surviving worms become established and mature (**plateau phase**). Upon maturation, the worms are expelled from the host by an immunological mechanism known as "self cure". The onset of the expulsion is sudden (between days 7 & 8 post infection) and the process is exponential (**loss phase 2**). Expulsion continues at a steady rate until a threshold is reached (**threshold phase**). A small residual population of worms are not expelled and survive for a prolonged period. Subsequently, the rats are relatively immune to reinfection (Jarrett *et al*, 1968).

1.6.2. Secondary infection

The pattern of establishment and expulsion during a primary infection is maintained in immune animals undergoing a secondary infection but there are quantitative differences. In a secondary infection, the **loss phase 1** is substantially greater than in a primary infection, the plateau phase is shorter (5 days long), and worms are rapidly expelled from the gastrointestinal tract on days 5 and 6 post secondary infection (Rothwell, 1988).

1.7. Immune responses during intestinal helminth infection

The discovery that T helper cells polarise into two different T helper cell subsets, Th1 and Th2 (Mosmann *et al*, 1989), has allowed a further refinement of the cell types responsible for the generation of different immune responses. Th1 and Th2 are characterised by the particular set of cytokines released. Th1 responses are associated with elevated levels of interferons, lymphotoxins and interleukins (IL)12

and 2. This results in the production of IgG2a antibody isotypes and a cell mediated immune response, whereas Th2 cells produce IL-4, IL-5, IL-9, IL-10 and IL-13, resulting in IgG1 and IgE antibody isotypes and a humoral response (Mosmann et al, 1989). It has now become clear that *N. brasiliensis* is a potent inducer of the Th2 type of the immune response, which gives rise to effector mechanisms leading to worm expulsion. This type of immune response is characteristic of host responses to pathogen invasion at mucosal sites and provides protection from intestinal helminths (Lawrence 2003).

In general, the different effector molecules that contribute to Th2 immunity are characterised by the accumulation of the Th2 subset of $CD4^+$ cells. These Th2 cells cannot kill parasites on their own; they rely on cytokine interleukin-mediated activation of innate effector cells. Th2 driven effector cells include granulocytic cells, such as mast cells, eosinophils, goblet cells and globule leukocytes (Abbas *et al*, 1996).

Mucosal mast cells (MMC) are associated with the production of IL-9 and are thought to be crucial effector cells in expulsion of nematode parasites, such as *Strongyloides venezuelensis* and *Trichinella spiralis* (Befus & Bienenstock, 1979; Abe & Nawa, 1988; Khan *et al*, 1993).

Associated with the number of MMC present in the gut is also the level of rat mast cell protease (RMCP II). RMCP II is a mediator which is localised exclusively in MMC (Gibson & Miller 1986) and is a serine proteinase. This enzyme is distinct from a chymotrypsin-like serine proteinase (RMCP I) in connective tissue mast cell

in its physical, biochemical and antigenic properties (Woodbury & Newlands, 1986). It has been reported that increased mucosal permeability is associated with expulsion of a primary *N. brasiliensis* infection, which coincides with maximal release of RMCP II (Nawa *et al*, 1979). It has also been suggested that RMCP II could have direct damaging effects on the nematode (Hyoh *et al*, 1999).

Eosinophils are present in the sub mucosa of the gastrointestinal tract and during an infection infiltration of these cells into the mucosa are particularly associated with the production of key cytokine IL-4 and IL-5 (Khan & Collins, 2004). However, their role in the expulsion of parasites is debatable. The use of blocking anti-IL-5 antibody did not appear to prevent the expulsion of a primary infection of *T. spiralis* (Herndon & Kayes, 1992). Therefore, the eosinophilia observed during a nematode infection may represent an immunopathological rather than a protective response and may merely be a consequence of the generalised inflammation induced by the Th2 response following infection (Lawrence, 2003). However, in general, these cells are not essential for the final steps in worm expulsion, including expulsion of *N. brasiliensis* (Kelly & Olgilvie, 1972, Urban *et al*, 1998).

The most recent evidence of a protective/expulsion role for inflammatory cells arrives from goblet cells. It is believed that final worm expulsion is goblet cell dependent (Nawa *et al*, 1994). There is a chronological association between goblet cell hyperplasia and worm expulsion in *N. brasiliensis* infections (Nawa *et al*, 1994). A major break-through in the determination of their role showed that there were alterations in the mucin glycoproteins present in the cells, suggesting that the more

mature goblet cells (acidic mucin) played a major role in worm expulsion (Ishikawa *et al,* 1994).

1.8. Immune response associated with *N. brasiliensis* expulsion

It is suggested that adult *N. brasiliensis* are expelled from the small intestine during a primary infection by a two step mechanism. Step 1 comprises of irreversible damage by the host immune responses, and reports have suggested that antibodies are responsible for the damaging of the worm. This is followed by step 2, comprising of the actual expulsive process of non-specific inflammatory responses, such as goblet cells, induced by the "damaged" worms (Ogilvie & Love, 1974). Once the parasites are cleared from the primary infection the animal retains a powerful degree of immunological memory (Kelly & Dineen 1972) and this memory manifests itself by an earlier expulsion in subsequent infections commencing around day 5 of re-infection (Olgilve & Love 1974).

As mentioned above, it is believed that antibodies play a vital role in damaging the worm in order for expulsion to occur (Ishiwata *et al*, 2002). It is known that primary intestinal nematode infections are typically accompanied by elevated IgE, IgA and IgG1 antibody isotypes (Onah & Nawa, 2000, Negrao-Correa *et al*, 1999). However, there is no evidence to indicate whether these antibodies represent a principal effector mechanism in resistance and worm expulsion during secondary infection and also in lactating animals experiencing a breakdown of immunity.

However, it is not known if the goblet cell response, or any other immune response associated with worm expulsion is sensitive to changes in scarce nutrient supply during a secondary infection, e.g. at times of a breakdown of immunity.

1.9. Nutritional sensitivity of immune resonses to gastrointestinal nematodes

The degree of nutritional sensitivity of the various immune responses associated with gastrointestinal nematode infections are still uncertain. Studies have indicated that chronic protein depletion decreases goblet cell number or mucin synthesis in rodent and pig small intestine (Neutra *et al*, 1974; Sherman *et al,* 1985). As well as goblet cells and mucosal mast cells, studies have looked at the effect of protein nutrition on globule leukocytes. After appropriate antigenic stimulation, mucosal mast cells release their contents and gradually develop into transitional cells and then into globule leukocytes (Huntley, 1992). The presence of an effect of protein supplementation on globule leukocytes has been observed in parasitised mammals (Houdijk *et al*. 2005). Earlier work carried out by Connan (1972) showed that in lactating rats the gut phase of a primary *N. brasiliensis* infection was protected from the animal's self-cure mechanism. This meant that the majority of worms persisted to day 34 when lactation was prolonged. When litters were removed at birth or when the suckling litter was reduced to three pups the rat was able to mount an immune response and reduce her worm burden. Connan (1972) believed that the prolonged worm burden might be due to the absence of an essential factor operating late in the self-cure mechanism, or to the presence of a potentiating factor which allows the worm to survive. In addition to this experiment, another study carried out by

Cummins et al, (1987) investigated the effect of feeding a protein deficient diet on immunological responses of the growing rat during a primary infection. They found that protein deficient rats were unable to expel their worm burden, unlike the protein sufficient rats. They also showed that protein deficient rats had fewer goblet cells and mucosal mast cells than the protein sufficient rats, indicating an impaired immune response.

As mentioned above, these experiments would suggest that various immune responses are sensitive to increases or reductions in dietary protein during a primary infection. However, there are no such studies investigating the nutritional sensitivity of the immune response to a secondary infection of *N. brasiliensis,* or at times of a breakdown of immunity during lactation. Therefore, there is a need for further work to be carried out to fully understand the nutritional sensitivity of the rodent model during a breakdown of immunity.

1.10. Nutritional sensitivity of resistance to *N. brasiliensis* in the rodent model

In their first experiments, Houdijk *et al* (2003) showed that a breakdown of immunity to *N. brasiliensis* occurs during re-infection in the lactating rat, but that this breakdown of immunity was not sensitive to protein supply, as the feeding of a high protein food did not result in a reduced breakdown of immunity to *N. brasiliensis*. However, they argued that the observed food intake of the high protein diets resulted in a protein supply that was approximately 25% lower than the protein

requirements suggested for lactating rats (National Research Council, 1995). Under those conditions, the increased protein supply would not have been expected to improve immunity to parasites (Coop & Kyriazakis, 1999) as these animals would not have achieved protein adequacy. Therefore, the experiment was repeated using foods with a higher protein content and lower gross energy content to ensure feed intake was increased (Friggens *et al*, 1993) and that consequently protein supply would not be scarce. Indeed, the results from the second experiment showed that lactating rats exhibit a breakdown of immunity to *N. brasiliensis*, and that this breakdown of immunity was sensitive to protein supply. Increasing the protein supply, resulted in a reduction in the number of eggs present in the colon and worm burden (Houdijk et al, 2005). However, this was confounded by an increase in feed intake *per se,* as high protein rats consumed nearly 3 times as much when compared to low protein rats. The increased food intake could have changed the gut environment into a less favourable one for parasite survival, mediated through effects of rate of passage or intake of specific non-protein food components. There is for example, evidence that fibre nutrition can affect gastrointestinal parasitism (Petkevicius *et al*, 1999). An increase in feed intake will have also resulted in an increase in all available nutrients and not only protein. Therefore, using these previous experiments, it was still not possible to determine whether it was indeed protein that affected the degree of breakdown of immunity to *N. brasiliensis*.

1.11 Thesis Objectives

The overall aim of this thesis was to use a rodent model to investigate the nutritional sensitivity of immunity to a nematode parasite (*N. brasiliensis*). With this in mind, the aim of the rodent model was to assess the sensitivity of the immune responses associated with a breakdown of immunity, in terms of parasitic burden and immunological analysis to protein, during lactation. This was achieved by undertaking a number of rat trials which are described in chronological order in the ensuing chapters. It was hypothesised that the rodent model would be sensitive to changes in MP availability, and that an increase in MP availability would reduced the animals parasitic burden. It was also expected that we would be able to identify the local immune responses associated with *N. brasiliensis* worm expulsion which are sensitive to these changes in MP availability.

The specific objectives of the thesis were:

1. To test the effects of changes in nutritional demand at a constant nutrient supply on the degree of immunity breakdown to *N. brasiliensis* in lactating rats. This was done in order to show the effects of changes in MP availabilty on nematode burden and immune responses, and rule out effects caused by changes in the gastrointestinal environment (Chapter Two).

2. To investigate whether changes in protein supply at a constant energy intake affect the resistance and immunity of lactating rats to *N. brasiliensis.* Under these conditions effects of protein supply could not be confounded with effects of any nutrient or energy intake. (Chapter Three).

3. To evaluate the effect of gradual increases in dietary protein supply on the resistance and immunity to *N. brasiliensis* in lactating rats. This was done as it was not known how gradual increments of scarce metabolisable protein actually affect milk production and the breakdown of immunity. (Chapter Four).

4. To test the effects of protein supply at different time points on the expression of immunity to *N. brasiliensis* during lactation in rats. This experiement was carried out as previous experiments found that on day 10 post secondary infection, immune responses associated with *N. brasiliensis* expulsion, were not sensitive to changes in MP availability. Therefore, we also sampled on day 5 and 15 post secondary infection (Chapter Five).

In summary, each individual objective of the thesis has helped to develop an experimental animal model of parasitism, which not only allowed the testing of our hypothesis, but could also be used for testing new hypothesis in the future.

Chapter Two

Effects of changes in nutritional demand at a constant nutrient supply on the degree of immunity breakdown to *Nippostrongylus brasiliensis*

2.1. Abstract

Lactating rats experience a breakdown of immunity to parasites, i.e. they carry larger worm burdens after re-infection compared to their non-lactating counterparts. Feeding high protein foods to lactating rats results in reduced worm burdens. This could be attributed to changes in gastrointestinal environment or to overcoming effects of nutrient scarcity on host immunity. The latter hypothesis was addressed through a manipulation of nutrient demand by manipulating litter size. Twenty-three rats were immunized prior to mating and re-infected on day 2 of lactation with 1,600 infective *Nippostrongylus brasiliensis* larvae. From parturition onwards, rats received *ad libitum* a low protein food (100 g CP/kg). Litter sizes were standardised to 9 (LS9), 6 (LS6) or 3 (LS3) pups, by day 2 of lactation. Ten days later, LS9 and LS6 rats carried more worms than LS3 rats. However, feeding treatments did not affect concentrations of mucosal inflammatory cells. Achieved feed intake did not differ consistently between the treatment groups. However, LS9 and LS6 rats lost weight, whilst LS3 rats gained weight during lactation. The results support the view that resistance to *N. brasiliensis* is sensitive to changes in nutrient demand, and the improved resistance to *N. brasiliensis* is likely due to effects of overcoming nutrient scarcity on host immunity.

2.2 Introduction

There has been renewed interest into why previously immune periparturient hosts are predisposed to a relaxation in immunity towards pathogens, including gastrointestinal parasites (Coop & Kyriazakis, 2001). The nutritional changes associated with pregnancy and lactation have been considered to be an important factor influencing this host-parasite relationship. It has been proposed that a reproductive animal prioritises the degree to which it allocates scarce nutrient resource(s) to various body functions (Coop & Kyriazakis, 1999). This nutrient-partitioning framework suggests that the allocation of scarce nutrients to the functions associated with parasite control have a low partial priority relative to reproductive effort. As a consequence, at times of nutrient scarcity, the periparturient animal would experience a breakdown of immunity to parasites, evidenced by an increased gastrointestinal nematode burden and faecal egg count.

The proposed nutritional basis of periparturient breakdown of immunity to parasites implies that the degree of parasitic burden would be affected by changes in the degree of nutrient scarcity. This hypothesis has been addressed in a rodent model. Earlier studies using previously naïve rats have shown that immune expulsion of a primary infection of the gastrointestinal nematode *Nippostrongylus brasiliensis* is impaired in lactating rats relative to their nulliparous controls (Dineen & Kelly, 1972). This model has now been extended to previously immune rats, demonstrating that lactating rats exhibit a breakdown of immunity to *N. brasiliensis*

(Houdijk *et al*, 2003a). A recent study has shown that this breakdown has indeed a nutritional basis, as feeding a high protein diet resulted in a reduced level of parasitism relative to a low protein diet (Houdijk *et al*, 2005a). However, it could not be excluded that this increased resistance was due to dietary components directly changing the gastrointestinal environment into a less favourable one for parasite survival, as offering the high protein diet also resulted in increased food intake *per se* (Houdijk *et al*, 2005a). There is evidence for example, that fibre nutrition can affect gastrointestinal parasitism through manipulation of the gut environment (Petkevicius *et al*, 1999). In addition to increasing nutrient supply, nutrient scarcity can also be reduced and eventually overcome through a reduction in nutrient demand, e.g. through manipulating (reducing) litter size. In recent studies on sheep, such manipulation has not resulted in differences in food intake (Houdijk *et al*. 2001 & 2006). Hence, this methodology may be used to study effects of reducing or overcoming nutrient scarcity on gastrointestinal nematode parasitism, whilst direct effects of diet on gut environment can be avoided.

It was hypothesised that the degree of *N. brasiliensis* infection occurring in the lactating rat offered a low protein food, would be sensitive to changes in nutrient demand. A reduction in nutrient demand, achieved through reducing litter size, would be expected to result in a reduced worm burden.

2.3. Materials and Methods

2.3.1. Animals and housing

Twenty three second parity female Sprague-Dawley rats (Harlan Ltd, Oxfordshire, UK) were housed in a room where ambient temperature was maintained at 21°C, relative humidity ranged from 45 to 65%, and artificial lighting was provided between 08.00-18.00 hours. Rats were housed in solid-bottomed cages with fresh sawdust being provided weekly and a handful of shredded plastic bubble wrapping for nesting material from 3 days before expected parturition until the end of the experiment. Wire-bottomed cages were used during mating and for faeces collection during the primary infection as described previously (Houdijk *et al*. 2003a). For mating, female rats were placed with a proven male breeder and mating was confirmed through the presence of a vaginal plug.

2.3.2. Foods

All rats were given *ad libitum* access to standard rat chow (222g digestible crude protein (CP) and 12.2 MJ digestible energy per kg dry matter (DM)), until mating was confirmed. Mated rats were given *ad libitum* access to a high protein food (210g CP per kg DM) for 10 days followed by a low protein food (60g CP/kg DM) until parturition. This feeding protocol was used to reduce body protein reserves during the second half of gestation in order to maximize the degree of protein scarcity

during lactation when rats are on low protein foods (Pine *et al*. 1994; Houdijk *et al*. 2005a). Parturition was considered as day 0 and from then until day 12 of lactation rats were given *ad libitum* access to a low protein food, formulated to supply 100g CP/kg DM. Ingredients and chemical analysis of the experimental food offered during lactation is shown in Table 1.

Table 1. Composition and analysis of the experimental food used during lactation

	Experimental Diet
Ingredients (g/kg fresh matter)	
Casein (plus 1% methionine)	103
Starch	304
Sucrose	152
Corn oil	197
Vitamins	47
Minerals	47
Cornflour	46
Choline	7
Lecithin	2
Alphacel	94
Analysed chemical composition (g/kg dry matter)	
Dry matter (g/kg fresh)	783
Gross Energy (MJ/kg DM)*	19.7
Crude protein	124
Ether extract	189
Ash	42

* Calculated from feed tables.

2.3.3. Infection protocol and experimental design

All rats were infected through a subcutaneous injection with *N. brasiliensis* according to a previously established protocol (Houdijk *et al*. 2003a). Rats received on day –37 (37 days before the realised mean parturition date) a primary infection of 1,600 third-stage infective larvae L_3 of *N. brasiliensis*, which were suspended in 0.5 ml sterile phosphate buffered saline. A secondary infection of 1,600 L_3 *N. brasiliensis* was administered on day 2 of lactation.

The experiment consisted of three treatments, with litter size being standardised on day 2 of lactation at 3 (LS3), 6 (LS6) or 9 (LS9) pups. The different litter sizes were chosen to result in different degrees of nutrient scarcity. Using previous data collected by Houdijk *et al* (2005a), it was hypothesised that a nursing dam fed the low protein food could support normal growth of 3 pups, resulting in nutrient abundance. However, when nursing 6 or 9 pups, achieved intake of the low protein food would limit her lactational performance, thus resulting in nutrient scarcity.

The aim was to obtain 7 replicates for each of the three treatments. However, 5 rats did not conceive, one was euthanised for unrelated reasons and data from one LS6 rat was omitted because litter size was not maintained at six pups. Therefore, the achieved number of replicates were $n = 6$ for LS9 and LS3, $n = 4$ for LS6.

All rats were killed on day 12 (i.e. ten days post secondary infection) for the assessment of worm burdens, number of nematode eggs in the colon contents, and concentration of inflammatory cells in the small intestinal mucosa (see below).

2.3.4. Body weight and food intake

Rats were weighed daily throughout the experiment. Feed intake was measured daily during gestation and lactation. The pups were counted and weighed daily from day 0. Foods offered during gestation & lactation were sampled during their preparation for the analysis of DM, crude protein (Kjeldahl-N x 6.25), ether extract and ash.

2.3.5. Nematode egg counts and worm burdens

From five days post primary infection (day -32), faeces were collected daily for 7 days as described previously (Houdijk *et al*. 2003a) for the assessment of faecal egg counts (eggs per g of faeces). This was done to provide evidence that a primary infection had established.

During the secondary infection, faeces were collected for three 24 h periods, starting in the morning of days 7, 9 and 11. On the first morning of faeces collection, the solid bottomed cages were cleaned and a small amount of fresh sawdust was added at 8 am. Fresh faeces were then collected every 2-3 hours until 6 pm. Half of the collected fresh faeces were kept refrigerated in a sealed plastic bag, pending the

assessment of faecal egg counts. The other half was used to determine its air dry matter content, which was estimated through drying at room temperature until the next morning. The rest of the faeces produced overnight were collected in the morning of days 8, 10 and 12, respectively. This enabled the total wet faeces production and nematode egg excretion over three 24-hour period to be calculated (see below). We needed to estimate total faeces production to account for eventual effects of faeces volume on faecal egg counts. The latter is a concentration measure, and the litter size treatments could have resulted in different volumes of faeces produced.

2.3.6. Colon contents and worm burden

Rats were sedated by gradually increasing ambient concentration of CO_2 and humanely killed by CO_2 asphyxiation on day 12 and dissected to collect the small and large intestine. Large intestinal contents were weighed and assessed for the concentration of nematode eggs, and nematodes were harvested from the small intestine, both as described before (Houdijk *et al*. 2003a). A small 2-cm section of small intestine, 15 cm down from the stomach, was placed in 4% paraformaldehyde for 6 h and then transferred to 70% ethanol. The small intestine sections were embedded in paraffin wax and mounted on slides for histochemical quantification of inflammatory cells. Sections of tissue were stained with toludine blue at pH 0.5 for the assessment of mucosal mast cells counts, whilst globule leukocytes and eosinophils counts were assessed after staining with carbol chromatrope and differentiation on morphological criteria (Huntley *et al*. 1995).

2.3.7. Calculations and statistical analysis

Due to their skewed nature, nematode egg excretion, number of eggs in the colon, nematode numbers, and inflammatory cell counts were transformed according to log (n + 1). This normalised the data before statistical analysis was carried out. These results are expressed as backtransformed means with 95% confidence intervals (CI). The non-transformed data are reported using the arithmetic mean values with their standard errors.

Nematode egg excretion during lactation (eggs/day) was calculated by multiplying the faecal egg count in the fresh faeces collected during the day (eggs/g) with the amount of fresh faeces produced over 24 h (g/day). The latter was calculated as the sum of the fresh faeces collected during the day and overnight: the latter was calculated from the dry faeces collected overnight and the estimated air dry matter content.

An one-way ANOVA was used to assess the hypothesis that the secondary infection of *N. brasiliensis* was sensitive to nutrient demand in lactating rats. The three levels of litter size were used to analyse the effects on nematode egg excretion, nematode eggs in colon, worm burden, and immune responses (mucosal mast cells, globule leukocytes, and eosinophils). The expectation was that parasitism would reduce, and immune responses increase with reduced litter size.

Effects of litter size on dam body weight, dam feed intake, and litter body weight, were assessed through repeated measure one-way ANOVA, taking into consideration the effect of time and interactions between litter size and time. Body weight at parturition was used as a covariate when analysing the effects of litter size on dam performance during lactation. All statistical analyses were performed using Genstat 6 for Windows (release 6.1, 2002; Lawes Agricultural Trust, Rothamsted, Herts., UK) and Minitab 12 (release 12.1, 1998; Minitab Inc.).

2.4. Results

2.4.1. Faecal egg counts during the primary infection and performance until parturition

All rats showed signs of a primary infection, and had started excreting nematode eggs by day -32. This then peaked at 52,479 (95% CI 46,880-58,748) eggs per gram faeces by day -29. The faecal egg counts then gradually decreased and by day$_{-26}$ all rats stopped excreting nematode eggs.

During the first 10 days of gestation, rats grew from 279 (SE 3.9) g to 315 (SE 5.1) g, with an average DM intake of 26.6 (SE 0.6) g/day. From then onwards and until parturition, the pregnant rats continue to grow to a mean weight of 349 (SE 9.1) g with an average DM intake of 18.3 (SE 0.1) g/day, which dropped to an average of 9.41 (SE 0.03) g/day just before parturition.

2.4.2. Food intake, dam and litter body weight during lactation

Figure 1a shows the DM intake for the dams during lactation. DM intake increased rapidly over time until day 3 of lactation (P=0.001), whilst litter size did not affect mean feed intake during the 12-day lactation period (P=0.37). However, litter size and time interacted for achieved feed intake (P=0.029) due to small differences in achieved food intake on two occasions. On days 10 and 12 only, DM intake of LS6 rats was lower than that of LS3 rats, whilst during the same days the intake of LS9 rats was intermediate to these groups (Figure 1a).

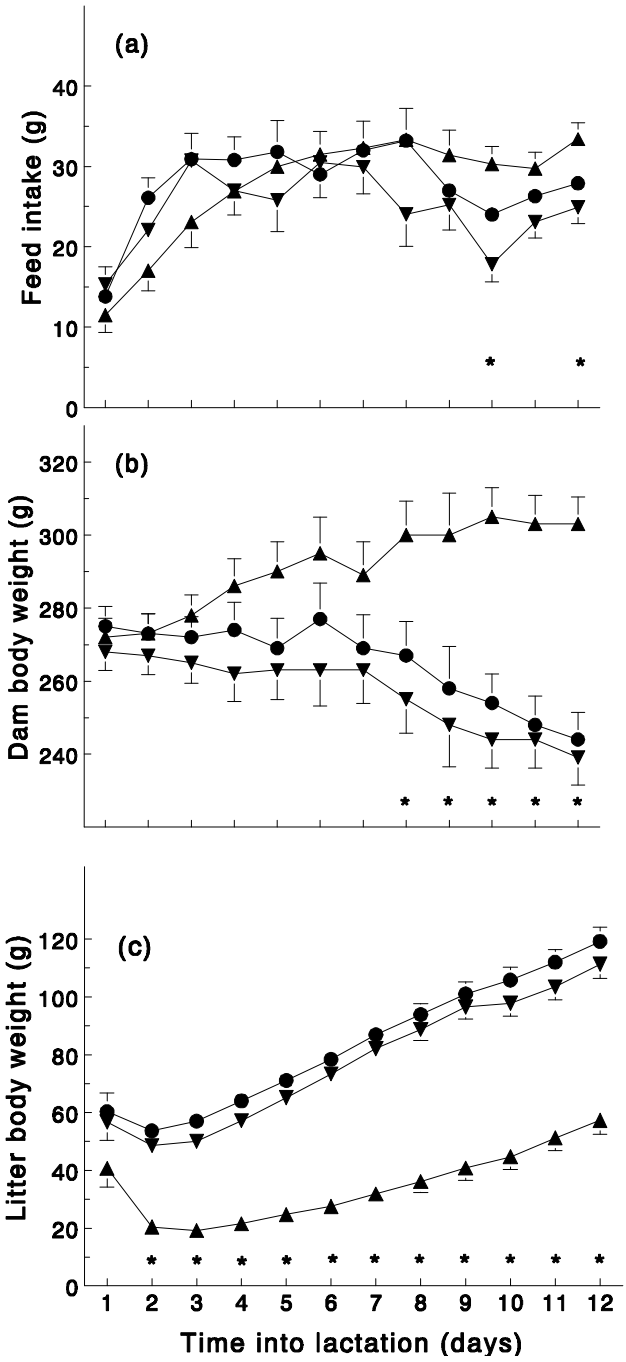

Figure 1: *Least square mean (with se) dam food intake (a), dam body weight (b) and litter body weight (c) of lactating rats, offered a low protein food (100g crude protein/kg DM) and nursing 9 (●), 6 (▼) or 3 (▲) pups. The * symbol denote effects of litter size at P<0.05 at specific time points.*

2.4.3. Dam performance

Figure 1b shows dam body weight during lactation. At parturition, mean dam body weight was 270 (SE 14.2) g. Litter size and time significantly interacted for dam body weight during lactation (P=0.001). LS9 and LS6 rats had smaller body weight than LS3 rats from day 5 onwards. Over the 12-day lactation period, LS9 and LS6 rats lost 24.0 and 24.6 g, respectively, whilst LS3 rats gained 35.2 g (s.e.d. 14.7 g; P=0.01).

2.4.4. Litter performance

Mean litter weight from day 0 during lactation is shown in figure 1c. At parturition, mean litter body weight was 53.1 (SE 2.8) g. Litter size and time significantly interacted for litter body weight during lactation (P=0.001). Litter size had a significant effect on mean litter weight from day 2 onwards, and this effect increased over time. Final litter weight did not differ between LS9 and LS6 rats. As expected, final pup weight increased with reduced litter size, and averaged 13.2, 18.5 and 20.3 g for LS9, LS6 and LS3 rats, respectively (s.e.d. 1.78 g; P=0.001).

2.4.5. Nematode egg excretion during lactation

Time and litter size each affected faeces production during lactation, but there was no interaction between time and litter size (P=0.46). Mean faeces production averaged 5.1, 3.3 and 3.5 g for day 7-8, 9-10 and 11-12 of lactation, respectively (s.e.d. 0.58 g; P=0.026), whilst it averaged 3.7, 3.4 and 4.7 g for LS9, LS6 and LS3,

respectively (s.e.d. 0.43 g; P=0.026). Effects of time and litter size on nematode egg excretion are therefore expressed as daily egg output to account for observed effects on faeces volume. Nematode egg excretion significantly increased over time from 1802 (729-4451), to 3791 (2257-6368) and 5826 (3909-8683) eggs per day for days 7-8, 9-10 and day 11-12, respectively (P=0.017). For the different litter sizes, nematode egg excretion averaged 3310 (2652-4132), 8438 (4021-17704) and 1731 (1278-2345) eggs per day for LS9, LS6 and LS3, respectively, but these differences failed to reach formal statistical significance (P=0.11).

2.4.6. Colon egg count and worm burden

The effects of litter size on the mean number of nematode eggs found in the colon, shown in figure 2a, were in a similar direction to the effect on worm burden (see below), but these effects were not significant (P=0.36). However, litter size had a significant effect on worm burden (P=0.01), as shown in figure 2b. Worm burden of LS9 and LS6 rats were similar but were both significantly larger than that of LS3 rats. Litter size did not affect the percentage of male and female worms (P=0.81); the percentage of male worms averaged 41.6 (SE 4.2) %.

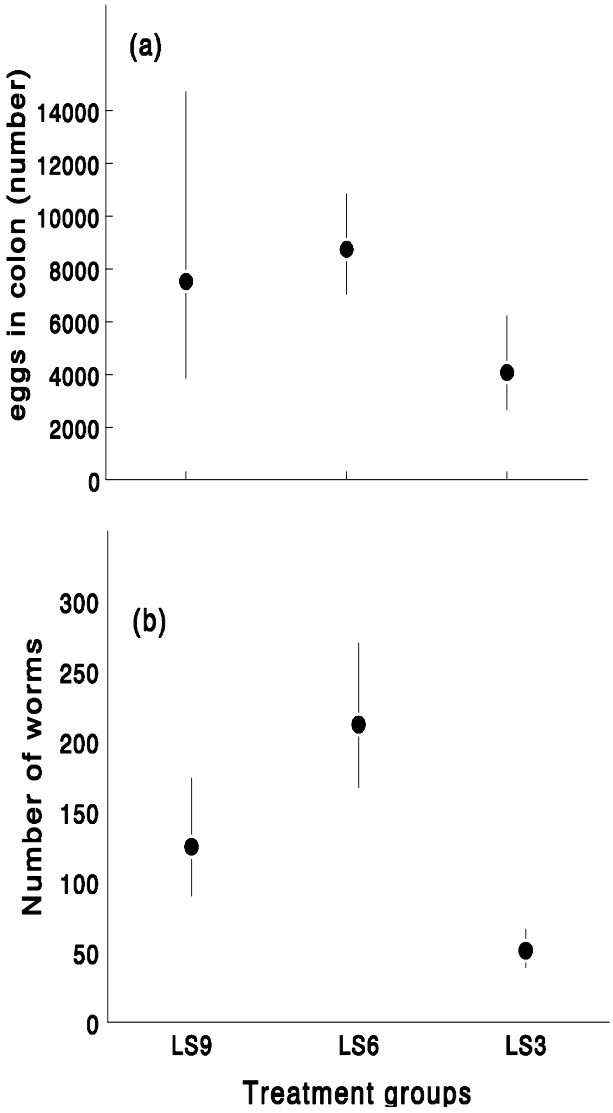

Figure 2. *Mean backtransformed (with 95% CI) number of eggs in colon contents (a) and worm burden (b) of lactating rats, offered a low protein food (100g crude protein/kg DM) and nursing 9 (LS9), 6 (LS6) or 3 (LS3) pups.*

2.4.7. Inflammatory cells

Feeding treatments had no significant effect (P=0.80) on the number of mucosal mast cells and eosinophils (P=0.13) in the small intestine. Overall, the mean concentration of mucosal mast cells was 51 (39-69) per mm^2, and that of eosinophils was 172 (147-205) per mm^2. Globule leukocytes were absent in all tested sections.

2.5. Discussion

At times of nutrient scarcity, the periparturient animal may experience a breakdown of immunity to parasites, evidenced by an increased gastrointestinal nematode burden and a faecal egg count. Houdijk *et al* (2005a) used a rodent model to assess whether a reduction in nutrient scarcity during lactation resulted in a reduced degree of parasitism. Feeding high protein foods resulted in a reduced worm burden, but this was confounded by the concomitant increase in food intake. Therefore, effects observed on parasitism may not necessarily have been associated with an increased nutrient supply, but could have been related to changes in the gut environment, as a consequence of increased food intake. The current experiment aimed to account for this uncertainty by testing the hypothesis that parasitism during lactation is sensitive to nutrient demand in the absence of changes in food intake. This would then further support the view that nutritional control of gastrointestinal parasitism may be associated with immunological changes in the gut environment.

Firstly, it is important to establish whether the current experiment had achieved the correct conditions under which the hypotheses could be tested. The hypothesis stated that worm burden during the periparturient period would be sensitive to changes in nutrient demand. This was to be evidenced by comparing the LS9, LS6, and LS3 feeding treatments. The aim was to create differences in nutrient demand, so that LS9 and LS6 rats would be in a nutrient scarce state while LS3 rats would achieve nutrient abundance. It is evident from the data obtained that these litter size treatments affected dam performance i.e., body weight change and litter gain (proxy for milk production). At similar levels of achieved food intake over the 12-day lactation period, LS6 and LS9 rats lost a significant amount of weight, whilst LS3 rats gained weight, and pup weight progressively increased from LS9 to LS3. Hence, the data support the view that LS6 and LS9 rats were indeed in a more nutrient scarce state than LS3 rats. Although it cannot be exclusively concluded from the current data, it might be argued that since LS3 rats were lactating and increasing in body size, they had indeed achieved nutrient adequacy. Hence, the hypothesis that the degree of *N. brasiliensis* infection is sensitive to differences in nutrient demand could be properly addressed in this experiment. The discussion will hereafter focus on the worm burden, as changes in the level of worm burden act as a reflection of how the host has coped with the parasite challenge. We focus on worm burdens rather than on nematode egg excretion and/or nematode eggs in colon because the worm burdens are much less sensitive to between-animal variation in achieved food intake/faeces production and/or colon content (e.g. Houdijk *et al*., 2003b, Athanasiadou *et al.*, 2001). These sources of variation have likely contributed, to a large extent, to the absence of significant feeding treatment effects on nematode egg

excretion and the number of nematode eggs in the colon contents (Fig 2). However, these effects were in the same direction and, on average, of the same magnitude as those on worm burdens.

The results from the present study show that at similar food intake over the 12-day lactation period used, a clear reduction in worm burden with reduced litter size was observed. Intuitively, one might have expected that the LS9 rats, and to a lesser extent the LS6 rats, would consume more of the low protein food than the LS3 rats, in order to compensate for the protein shortage relative to the extra demand placed on them. However, the low protein to energy ratio of food used may have prevented an increased food intake to satisfy protein requirements, because of the metabolic consequences that would arise from the associated excess energy intake (Friggens *et al*. 1993). The observed reduction in worm burden at similar achieved food intake indicates an improvement of host resistance in the absence of direct changes in gut environment. Therefore, this supports the view that the nutritionally achieved reduction in worm burden in our experiment, as well as in Houdijk *et al* (2005a), is likely achieved through effects of nutrient availability on expression of immunity, and not through changes in the gastrointestinal environment.

The observation that LS3 rats had significantly smaller worm burdens than LS6 and LS9 rats, is consistent with results obtained from farm animal studies. Parasitised single-rearing ewes have usually lower faecal egg counts and smaller worm burdens than their twin-rearing counterparts (Houdijk *et al* 2001; Donaldson *et al*. 1998; Kahn *et al*. 2003), whilst similar effects have been seen in low-yielding dairy goats

compared to high producing goats (Chartier *et al*. 2000). In addition, the effects of nutrient supply on worm burdens in the *N. brasiliensis* infected lactating rat (Houdijk *et al*. 2005a) are also consistent with a large number of farm animal studies (e.g. Houdijk *et al*. 2003b; Donaldson *et al*. 1988; 2001; Kahn *et al*. 2003). Hence, the evidence obtained to date shows that our *N. brasiliensis* lactating rat model can reproduce similar nutritional sensitivities of host resistance to gastrointestinal nematode parasites during the periparturient period as observed in other lactating hosts. This supports the view that this model may be used to assess nutritional sensitivity of periparturient immune responses that may be associated with nutritionally improved resistance to parasites.

In the current experiment, we investigated some of the possible underlying immune responses that may be associated with nutritionally improved resistance to *N. brasiliensis* during lactation, through the measurement of specific inflammatory cells. Mucosal mast cells have long been thought to be important effectors cells in expulsion of nematode parasites in general, and this role has been well documented in infections with *N. brasiliensis* (Befus & Bienenstock, 1979). However, more recent studies have suggested that the final phase of immune expulsion of *N. brasiliensis* in primary infections is mast cell independent (Nawa *et al*, 1994), although a role for mucosal mast cells in secondary infections may not be excluded (Katona *et al*, 1988). The current view is that mast cells may play a role in leading to the outcome of the immune response, rather than serve simply as late-stage effector cells involved with the actual immune expulsion of the parasite (Maizels & Holland, 1998, Maizels *et al*, 2004). Previous work indicated that dietary protein deficiency

in growing rats resulted in delaying the expulsion of *N. brasiliensis* from the gastrointestinal tract and that this was associated with a reduction in the number of mucosal mast cells compared to protein-replete controls (Cummins *et al*, 1987). This points towards at least some degree of nutritional sensitivity of the mast cell response to *N. brasiliensis* infection. Here, we compared the number of mucosal mast cells and eosinophils in the different treatment groups, on the basis that the activity of the latter being influenced by the former (Capron *et al*. 1978). Our analysis showed that reducing nutrient demand had no effect on either parameter. Future work will expand upon the immunological measurements to include isotype-specific humoral responses and the release of cell-specific factors such as mast cell proteases, to provide additional measures of cellular activity. It may also be possible to expand the immune response analysis by accounting for goblet cells. It is known that these cells undergo dramatic expansion in the parasitized gut epithelium. Evidence has accumulated to implicate goblet cells and their mucus production as key elements in the expulsion of *N. brasiliensis* during a primary infection. High levels of mucus production may trap parasites in the lumen and minimise their ability to attach in the gut (Nawa *et al*, 1994). However, whether goblet cells are also involved in immune expulsion of secondary *N. brasiliensis* infections, as in our lactating rat model, remains to be investigated.

Globule leukocytes were absent in mucosal tissues collected in this current experiment. A possible explanation as to why no globule leukocytes were found could be related to the timing of sampling used. Connan (1973) found globule leukocytes from samples obtained after more than ten days of a single secondary

infection with *N. brasiliensis*. Another reason could be due to the mode and short period of infection. In nature, both the primary and secondary infection would be a gradual, trickle one, acquired over a period of time. The presence of an effect of protein supplementation on globule leukocytes has been consistently observed in other parasitized hosts, where a gradual, trickle infection has been used (Houdijk *et al*. 2005b).

In conclusion, the results of this experiment support the view that the extent of *N. brasiliensis* infection during the periparturient period is sensitive to changes in nutrient demand, and that these effects are independent of changes in the gastrointestinal environment. This supports the view that nutritional control of resistance to parasites during lactation is mediated through host immunity. This rodent model can now be used to fully understand the underlying mechanisms of the nutritional basis of relaxation in immunity during the periparturient period.

Chapter Three

Changes in protein supply and nutrient demand affect resistance and immunity of lactating rats to *Nippostrongylus brasiliensis*

3.1 Abstract

Reducing protein scarcity through increased protein supply or reduced nutrient demand would be expected to increase resistance to parasites during the periparturient period. This hypothesis was addressed in a lactating rat model, as lactating rats show a breakdown of immunity to the intestinal nematode *Nippostrongylus brasiliensis*. Eighteen rats were given a single dose of 1600 *N. brasiliensis* larvae prior to mating (primary infection), and re-infected with the same dose on day 2 of lactation. During lactation, rats received a low protein diet (LP, 100g CP/kg DM) or a high protein diet (HP, 300g CP/kg DM). Rats were fed restrictively at 7.5% of their parturition body weight. Litter sizes for LP groups were standardised to 9 (n=6) or 3 (n=6) pups, while HP groups had 9 pups (n=6). Rats were slaughtered on day 12 to assess the concentration of nematode eggs in colon contents. Mucosal scrapings were taken to assess local antibodies (IgA, IgE, IgG_1 and IgG_{2a}) and rat mast cell proteases (RMCP-II). Feeding treatments had a significant effect on the number of eggs found in the colon contents. HP9 and LP3 rats had significantly lower number of eggs in their colon than LP9 rats. HP9 and LP3 rats tended to have higher levels of local antibodies and RMCP-II than LP9 rats; the trend was the mirror image of the significant effect of treatment on the number of eggs in the colon. The results support the view that the periparturient breakdown of immunity to *N. brasiliensis* is sensitive to changes in protein scarcity.

3.2. Introduction

During the periparturient period, the expression of acquired immunity to parasites usually breaks down (Houdijk *et al*, 2005b); for gastrointestinal parasites this is characterised by an increased worm burden and elevated excretion of eggs into the environment. The breakdown of immunity plays an important role in parasite epidemiology, as the parasitized periparturient animal can be a major source of infection for their parasite-naïve offspring. It is thought that this relaxation in immunity during pregnancy and lactation is associated with the changes in nutrient demand and utilisation that occur during this period. It has been proposed that a reproducing mammal prioritises the degree to which it allocates scarce nutrient resources to various body functions (Coop & Kyriazakis, 1999). This nutrient-partitioning framework suggests that the allocation of scarce nutrients to the functions associated with parasite control have a lower partial priority than those associated with the reproductive effort. As a consequence, at times of nutrient scarcity, which often occurs during the periparturient period, hosts would experience a breakdown of immunity to parasites.

The proposed nutritional basis of periparturient breakdown of immunity to parasites implies that the degree of parasitic burden would be affected by changes in the degree of nutrient scarcity. This hypothesis has been addressed in a rodent model, as it has been demonstrated that lactating rats exhibit a breakdown of immunity to

Nippostrongylus brasiliensis (Houdijk *et al*, 2003a). Normanton *et al*, (2006) have shown that overcoming nutrient scarcity through manipulating litter size, resulted in reduced worm burdens; lactating rats nursing 3 pups harboured fewer worms than those nursing 6 or 9 pups when offered *ad libitum* the same, low protein food. Houdijk *et al* (2005a) have also shown that feeding high protein foods *ad libitum* during lactation results in a reduced worm burden, when compared to lactating rats receiving low protein foods. However, in both experiments, effects could have been confounded with increased food intake *per se* and a consequent increase in energy or any other nutrient intake. An increase in dietary components could also have directly changed the gastrointestinal environment into a less favourable one for parasite survival (Petkevicus *et al*, 1999).

Although these studies support the view that the breakdown of immunity to *N. brasiliensis* has a nutritional basis, the underlying immune responses that may be associated with nutritionally improved resistance to *N. brasiliensis* during lactation still need to be identified. It is known that *N. brasiliensis* is a potent Th2 inducer, which gives rise to effector mechanisms leading to worm expulsion, such as isotype-specific humoral responses (Maizels et al, 2004). It is known that intestinal nematode infections are typically accompanied by elevated IgE, IgA and IgG_1 antibody isotypes (Onah & Nawa, 2000), although there is very little consistent and convincing data to indicate them as principal effector mechanisms in resistance to intestinal nematodes. As well as humoral responses, several studies have demonstrated that gastrointestinal nematode infections are invariably accompanied by inflammatory cell responses, such as an increased concentration of mucosal mast

cells , rat mast cell protease (RMCP II), eosinophils, goblet cells and globule leukocytes (Miller, 1996). However, it is unclear what role these cells play in the elimination of the adult parasite, especially during a secondary infection, and whether they are sensitive to changes in host nutrition.

The objective of the current experiment was to test the effects of increased protein supply or reduced nutrient demand on the resistance to parasites and immune responses in lactating rats. To avoid the previously observed changes in food intake associated with manipulation of dietary protein contents (Houdijk *et al*, 2005) and nutrient demand (Normanton *et al*, 2006), experimental foods were offered in restricted amounts. It was hypothesised that the degree of *N. brasiliensis* infection occurring in the lactating rat under a restricted feeding regime would be sensitive to changes in protein scarcity. A reduction in nutrient demand, achieved through reducing litter size, and an increase in protein supply, achieved by feeding a high protein diet, would be expected to result in reduced parasite burdens and affect local immune responses thought to be involved during expulsion of the nematode from the host.

3.3. Materials and Methods

3.3.1. Animals and housing

The experiment was carried out under a Home Office licence (PPL 60/2616) allowing for experimental infection and feeding low protein foods. Eighteen second

parity female Sprague-Dawley rats (Harlan Ltd, Oxfordshire, UK) were housed in a room where ambient temperature was maintained at 21°C, relative humidity ranged from 45 to 65%, and artificial lighting was provided between 08.00-18.00 hours. Rats were housed in solid-bottomed cages with fresh sawdust being provided weekly and a handful of shredded plastic bubble wrapping for nesting material from 3 days before expected parturition until the end of the experiment. Wire-bottomed cages were used during mating and for faeces collection during the primary infection as described previously (Houdijk *et al*. 2003a). For mating, female rats were placed with a proven male breeder and mating was confirmed through the presence of a vaginal plug.

3.3.2. Foods

All rats were given *ad libitum* access to standard rat chow until mating was confirmed. Mated rats were then given *ad libitum* access to a high protein food (210g CP per kg DM) for 10 days followed by a low protein food (60g CP/kg DM) until parturition. This feeding protocol was used to reduce body protein reserves during the second half of gestation in order to maximize the degree of protein scarcity during lactation when rats are on low protein foods (Pine *et al*. 1994; Houdijk *et al*. 2005a).

Parturition was considered as day 0 and from then until day 12 of lactation rats were fed restrictively (7.5% of parturition body weight) one of two foods: low protein (LP), formulated to supply 100g CP/kg DM or a high protein (HP), formulated to

supply 300g CP/kg DM. The level of restricted feeding used was based on the mean achieved dry matter intake and parturition body weight of the low protein rats from a previous experiment (Normanton *et al*, 2006). The diets were made to the same specification as the previous experiment, so that the 7.5% level was expected to lead to the absence of refusals, and hence similar intake between treatment groups. The different diets were achieved through the iso-energetic exchange of casein against digestible carbohydrates and oil, which resulted in both diets containing 19.7 MJ gross energy per kg DM. Ingredients and chemical analysis of the experimental foods offered during lactation are shown in Table 1.

Table 1: Composition and chemical analysis of the experimental foods used during lactation.

Ingredients (g/kg fresh matter)	Experimental Diets Low Protein, (LP)	High Protein, (HP)
Casein (plus 1% Methionine)	103	307
Starch	304	206
Sucrose	152	102
Corn oil	197	140
Vitamins	47	47
Minerals	47	47
Cornflour	46	46
Choline	7	7
Lecithin	2	2
Alphacel	94	94
Analysed chemical composition (g/kg dry matter)		
Dry matter (g/kg fresh)	744	644
Gross Energy (MJ/kg DM)*	19.7	19.7
Crude protein	118	320
Ether extract	162	133
Ash	51	49

* Calculated from feed tables

3.3.3. Infection protocol and experimental design

All rats were infected with *N. brasiliensis* according to a previously established protocol (Houdijk *et al*. 2003a). Rats received on day$_{-35}$ (35 days before the realised mean parturition date) a primary infection of 1,600 third-stage infective larvae L_3 of *N. brasiliensis*, which were suspended in 0.5 ml sterile phosphate buffered saline. A secondary infection of 1,600 L_3 *N. brasiliensis* was administered on day 2 of lactation.

The experiment consisted of three treatments; LP3, LP9 and HP9. The litter size of the LP rats was standardised on day 2 of lactation at 3 (LP3) or 9 (LP9) pups, whilst the litter size of HP rats was standardised at 9 pups (HP9). The different litter sizes in LP were chosen to result in either nutrient scarcity (LP9) or nutrient abundance (LP3). It was expected that LP9 rats would have a larger parasitic load compared to LP3 and HP9 rats, but that HP9 rats would have the same, low parasite load as LP3 rats. This is because even though they would have considerably larger protein demand, the high protein content of their diet should balance out this difference. Smaller litter sizes were not included within the HP treatment as each treatment would have been in a protein adequate state.

All rats were killed on day 12 (i.e. ten days post secondary infection) for the assessment of the number of eggs in the colon. This was used to assess the degree of

parasitism, as the standard protocol for worm burden collection does not allow for simultaneous collection of sufficient mucosal scrapings in order measure immune responses.

3.3.4. Body weight and food intake

Rats were weighed daily throughout the experiment. Intake was measured daily during gestation and any refusals that did occur during lactation were collected every morning and weighed. The pups were counted and weighed daily from day 0. Foods offered during gestation & lactation were sampled during their preparation for the analysis of DM, crude protein (Kjeldahl-N x 6.25), ether extract and ash.

3.3.5. Nematode egg counts

Five days post primary infection (day_{-30} relative to parturition) faeces were collected daily for five days as described previously (Houdijk *et al*. 2003a), for the assessment of faecal egg counts (eggs per g of faeces). This was done to provide evidence that a primary infection had established and immunity was developed.

3.3.6. Nematode egg count during the secondary infection

All rats were sedated and humanely killed by CO_2 asphyxiation on day 12 and dissected to collect the small and large intestine. Large intestinal contents were collected, weighed and assessed for the concentration of nematode eggs as described before (Houdijk *et al*. 2003a). Worm burdens were unable to be collected as the small intestine used for the collection of worms was needed for the collection of mucosa, as described below. A small 2 cm section of small intestine, 15 cm down from the stomach, was placed in 4% paraformaldehyde for 6 h and then transferred to 70% ethanol. The small intestine sections were then processed for the measurement of the concentration of mucosal mast cells, globule leukocytes, and eosinophils as described previously (Huntley *et al*. 1995).

3.3.7. Mucosal scrapings

Mucosa samples were obtained by scraping approximately 15cm of the anterior small intestine with a glass microscope slide. Samples were then transferred into a bijoux tube and frozen. For the analysis of antibody and rat mast cell protease (RMCP II), the samples were thawed and homogenised on ice, using ice-cold phosphate buffered saline (PBS), to solubilise the mucosa sample.

3.3.8. Analysis of mucosa samples for soluble antibody

An enzyme linked immunosorbent assay (ELISA) method was used for the detection of the antibody isotypes IgG1, IgG2a, IgA and IgE in mucosal tissue. High binding ELISA plates were coated with 10µg/ml of adult *N. brasiliensis* antigen, in 50 µl/well bicarbonate buffer pH9.6 for 1 hour. Plates were then washed three times with PBS/Tween 20/NaCl solution. Mucosal homogenates for each rat were used in dilution 1 in 10 in PBS. These were added in 50 µl/well PBS and incubated for 1 hour at room temperature, following which plates were washed six times as above. Isotype-specific secondary antibodies were then added (Serotec, UK). All secondary antibodies were diluted in PBS/Tween 80 at a 1/1000 dilution and incubated on the plates for 1h at room temperature. Following washing six times as above, horse radish peroxidase (HRP) conjugated anti-mouse immunoglobulin antibody (Serotec, UK) was added to each well (50µl). This was again incubated for 1 hour. Plates were then washed six times as above, and then the antibody isotypes could be detected by the addition of 50µl/well of Sigma Fast-OPD and the colour allowed to develop for around 15 minutes before stopping with 2.5 M H_2SO_4. Following colour development, optical density values (OD) for each well were read at 492 nm using a plate reader.

3.3.9. Analysis of mucosa samples for RMCP II

Rat mast cell protease II (RMCP II) levels were detected and quantified from mucosa by ELISA as described by Miller et al (1983), using a commercially available kit

(Moredun Scientific Limited, RMCP II ELISA). Assays were carried out according to manufacturer's instructions. ELISA plates were coated with 2 µg/ml anti-RMCP II monoclonal antibody in bicarbonate buffer pH 9.6 at 4°C overnight. Following six washes with phosphate buffered saline with Tween-20 (PBST) plates were blocked for unspecific protein at 37°C for 30 min with 4% Bovine serum albumin in PBST. Plates were washed again three times and standards in series 0.5-12 ng/ml of RMCP II in PBST were added in. Mucosal homogenates were then plated in four dilution series of times ten, (1:10, 1:100, 1:1000, and 1:10,000) and incubated at 37°C for 30 min. Following a further six washes, plates were incubated for 1h with 50µl/well conjugated antibody solution at 37°C. Plates were once again washed six times before addition of 50 µl/well substrate (specific to the RMCP II kit) to allow for the colour formation. Colour formation was allowed to progress for around 15 min before stopping with 0.25M H_2SO_4. OD values for each well were then read at 450 nm on a microplate reader and RMCP II levels calculated by comparison against a plotted standard curve.

3.3.10. Calculations and statistical analysis

Due to their skewed nature, colon egg count, inflammatory cell counts, antibody analysis, and RMCP II levels were transformed according to log (n+1) to normalize data before statistical analysis. The transformed data are reported as backtransformed means, accompanied by a backtransformed lower and upper limit. These were calculated via 10^a-1 where $a = \mu+0.5\times\sigma^2$ (Johnson et al., 1988), with μ, μ - s.e. and μ + s.e. as the mean, lower and upper limit of the transformed data, and σ as their

standard deviation. Consequently, backtransformed lower and upper limits characterise an unequally distributed range around the backtransformed means.

One-way ANOVA was used to assess the hypothesis that the secondary infection of *N. brasiliensis* was sensitive to an increased protein supply or reduced nutrient demand in lactating rats. The 3 levels of feeding treatments were used to analyse the effects on nematode egg excretion, nematode eggs in colon, and immune responses (inflammatory cells, antibodies, and RMCP II). We tested the effect of assumed protein scarce state (LP9) vs assumed protein sufficient state (HP9), and for assumed nutrient abundancy (LP3) vs assumed nutrient scarcity (LP9). The expectation was that parasitism would reduce and immune responses increase with reduced litter size and increased protein supply.

For the immune responses, we also used contrast statements to test the effect of assumed protein scarce state (LP9) vs assumed protein sufficient state (HP9 & LP3). This was achieved through two orthogonal contrasts under a one-way ANOVA using all three feeding treatments. The two contrasts compared HP9 with LP3, and also compared combined HP9 and LP3 with LP9. The expectation was that the level immune responses would be similar between HP9 and LP3, but that the combined HP9 and LP3 would have higher immune responses compared with LP9.

Effects of feeding treatments on dam body weight, dam feed intake, and litter body weight were assessed through repeated measure one-way ANOVA, taking into consideration the effect of time and interactions between feeding treatments and

time. Body weight at parturition was used as a covariate when analysing the effects of treatments on maternal weight during lactation. All statistical analyses were performed using Genstat 6 for Windows (release 6.1, 2002; Lawes Agricultural Trust, Rothamsted, Herts., UK) and Minitab 12 (release 12.1, 1998; Minitab Inc.).

3.4. Results

3.4.1. Faecal egg counts during the primary infection and performance until parturition

All rats showed signs of a primary infection by excreting nematode eggs from day$_{-30}$. This then peaked at 40,220 (95% CI 35,259 – 48,879) eggs per gram by day$_{-27}$. The faecal egg counts then gradually decreased and by day$_{-25}$ all rats stopped excreting nematode eggs.

During the first 10 days of gestation, rats grew from 275g (SE 4.2) to 308g (SE 5.9), with an average DM intake of 21.3 g/day (SE 0.5). From then onwards and until parturition, the pregnant rats continued to grow to a mean weight of 364g (SE 9.7) with an average DM intake of 15.8g/day (SE 0.1), which dropped to an average of 7.4g (SE 0.03) on the day before parturition.

3.4.2. Food intake, dam and litter body weight during lactation

Figure 1a shows the DM intake for the dams during lactation. The data is shown from day 2, after litter size standardisation. Feeding treatment and time did not significantly interact for DM intake (P=0.304). Treatments imposed did not affect mean feed intake over the 10 day lactation period (P=0.33).

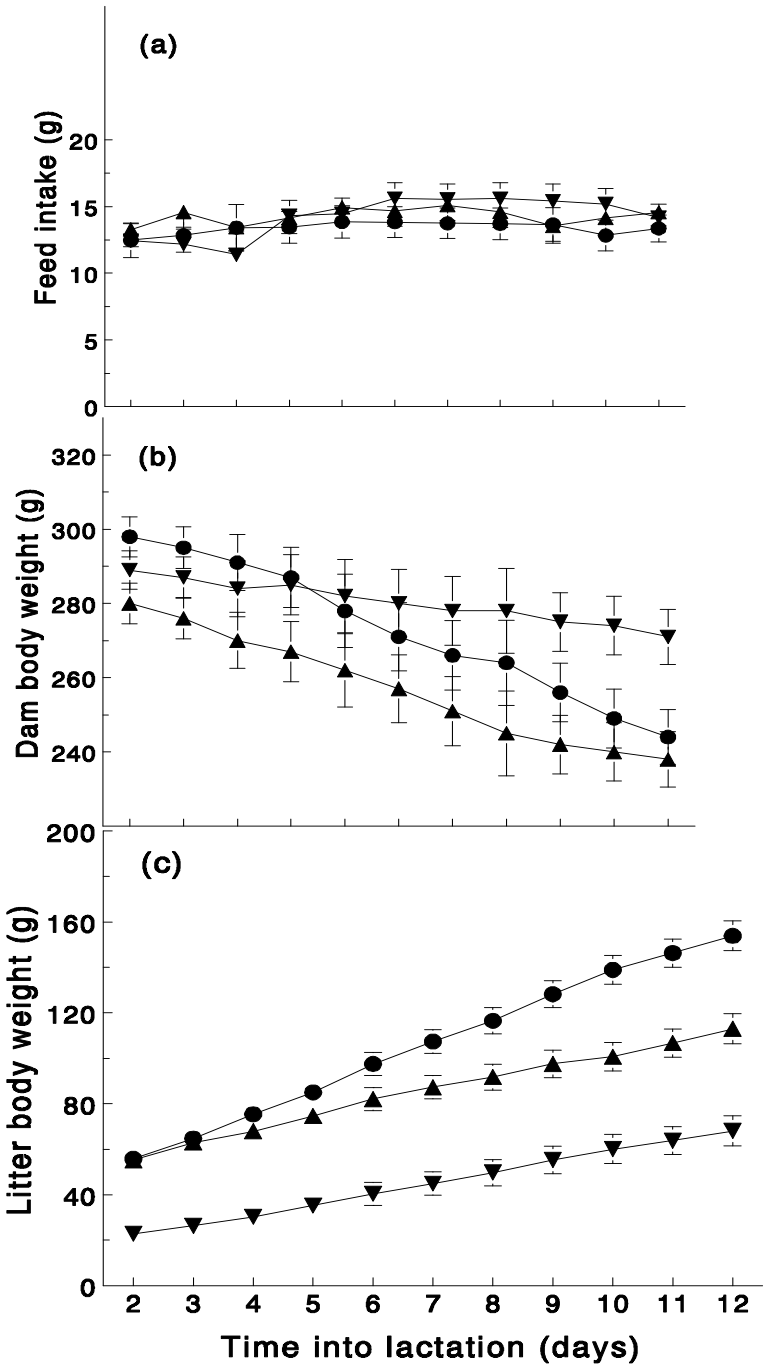

Figure 1: Dry matter dam food intake (with se) (a), least square mean (with se) dam body weight (b) and litter body weight (c) of lactating rats, restrictedly offered high protein foods (300g crude protein/kg DM) and nursing 9 pups (●), or low protein foods (100g crude protein/kg DM) and nursing 3 (▼), or 9 (▲) pups.

3.4.3. Dam performance

Figure 1b shows dam body weight during lactation. At parturition, mean dam body weight was 293 g (SE 7.6). There was no difference between treatment groups. By day 2 there was already a difference in dam body weight, but this was not significant. Feeding treatment and time significantly interacted for dam body weight during lactation (P=0.001). This was reflected in differences in body weight loss. HP9 rats lost 42g, LP9 rats lost 50g and LP3 rats lost 15g over the 12 day lactation period. HP9 rats did not differ significantly from LP9 rats in dam body weight (P=0.46, SE 12.8), while LP3 rats were significantly heavier than both HP9 and LP9 rats (P=0.001) at the end of lactation.

3.4.4. Litter performance

Mean litter weight from day 2 during lactation is also shown in Figure 1c. At parturition, mean litter weight was 53 g (SE 6.5) and there was no difference between treatment groups. However, feeding treatments had a significant effect (P=0.001) on mean final litter weight. HP9 litter gain (98g) was significantly higher (P=0.001) than the LP treatments. LP9 litter gain (58g) was significantly higher than LP3 (45g) litter gain (P=0.047). Feeding treatments also had a significant effect (P=0.001) on individual pup weight. Final LP3 pup weight (22.7g) was significantly higher than LP9 pup weight (12.5g) and HP9 pup weight (17.10g), (P=0.001, SE 6.59).

3.4.5. Colon egg count

The effect of feeding treatments on backtransformed mean number of eggs in the colon is shown in figure 2. LP9 egg count was significantly higher than that of LP3 (P=0.001), and HP9 (P=0.006). The mean number of eggs found in the colon for HP9 was significantly higher than LP3 (P=0.001).

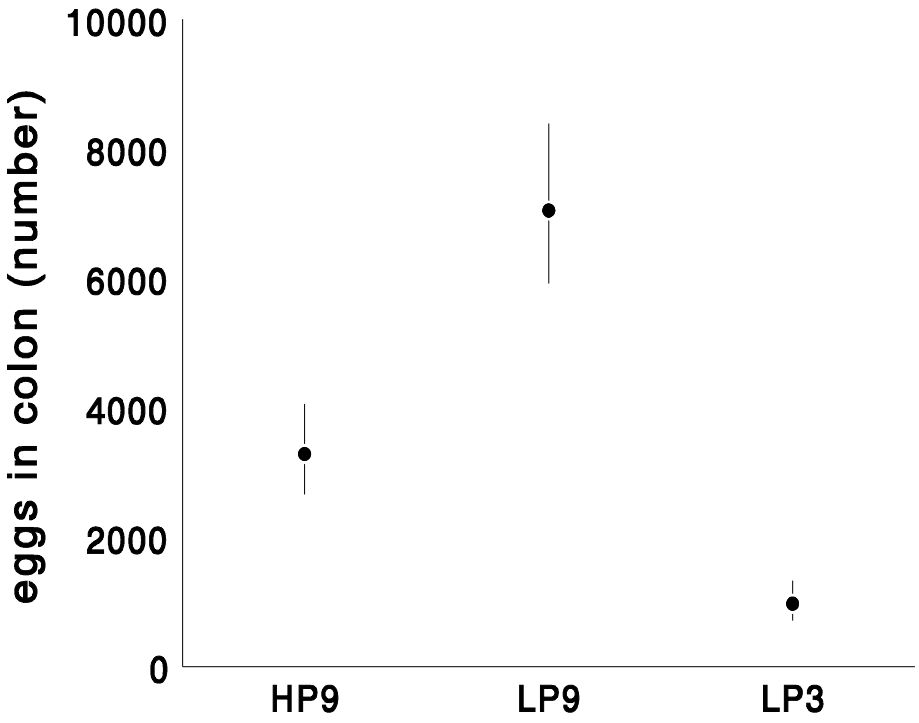

Figure 2. Mean backtransformed (with backtransformed standard errors) number of eggs in colon contents of lactating rats, restrictedly offered high protein foods (300g crude protein/kg DM) and nursing 9 pups (HP9), or low protein foods (100g crude protein/kg DM) and nursing 3 (LP3) or 9 (LP9) pups.

3.4.6. Immunological analysis

Treatment groups had no significant effect (P=0.88) on the number of mucosal mast cells and eosinophils (P=0.62) in the small intestine mucosa. Overall, the mean concentration of mucosal mast cells was 33 (14-71 95% CI) per mm^2, and that of eosinophils was 145 (81-241 95% CI) per mm^2 . Globule leukocytes were absent in all tested sections.

As shown in figure 3, LP3 rats and to a lesser extent HP9 rats, displayed a trend to have higher immunoglobulin levels compared to LP9 rats. The trend was the mirror image of the effect of treatment on the number of eggs in the colon. However, these results failed to reach statistical significance, IgA (P=0.243), IgE (P=0.227), IgG_1 (P=0.244) and IgG_{2a} (P=0.224). The contrast HP9 + LP3 compared to LP9 showed a tendency for HP9 + LP3 rats to have higher levels of immunoglobulins than LP9 rats IgA P=0.110, IgE P=0.093, IgG_1 P=0.144, and IgG_{2a} P=0.113.

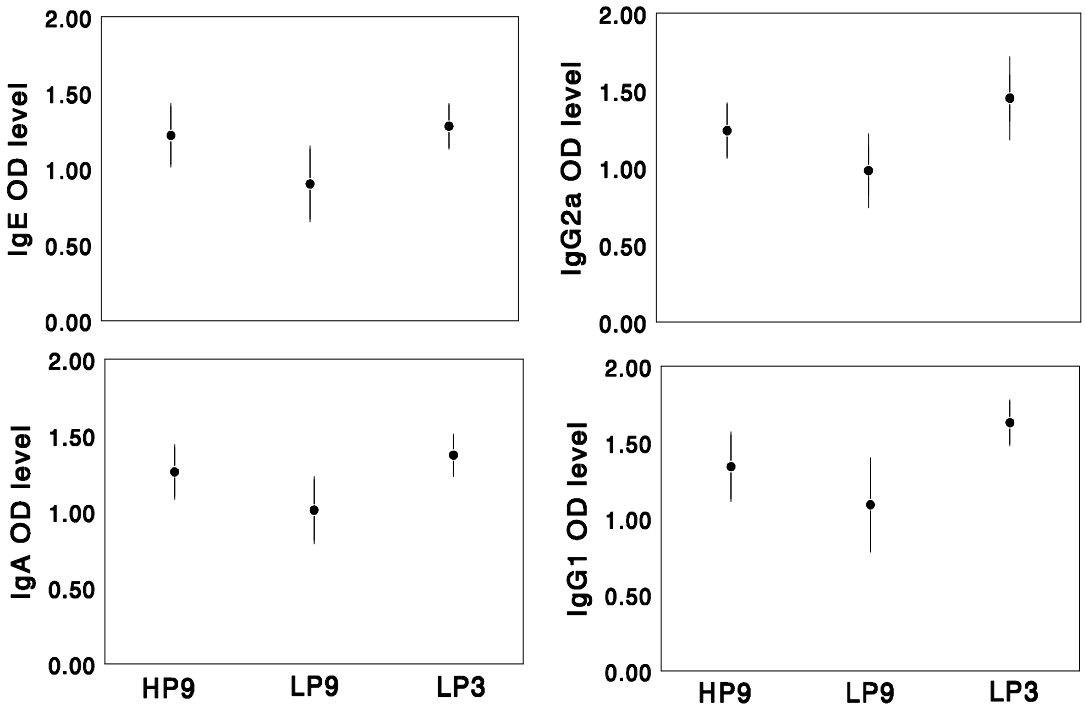

Figure 3. Mean backtransformed (with backtransformed standard errors) OD levels of IgA, IgE, IgG1 and IgG2$_a$ from lactating rats, restrictedly offered high protein foods (300g crude protein/kg DM) and nursing 9 pups (HP9), or low protein foods (100g crude protein/kg DM) and nursing 3 (LP3) or 9 (LP9) pups.

Although feeding treatments did not significantly affect the level of RMCP II present in the mucosa (P= 0.56), there was a trend for LP3 (6.73 ng/ml, 5.94-7.57 95% CI) and HP9 (6.40 ng/ml, 4.99-8.01 95% CI) rats to have a higher concentration compared with LP9 (6.11 ng/ml, 4.18-8.54 95% CI). The contrast HP9 + LP3 compared to LP9 showed a trend for HP9 + LP3 to have higher levels of RMCP II than LP9 rats. RMCP II levels tended to be positively correlated with the number of mucosal mast cells but this correlation did not approach statistical significance (r = +0.3, P=0.282).

3.5. Discussion

Recent studies on lactating rats have shown that during the periparturient period, a significant reduction in gastrointestinal parasitism can be achieved by either increasing nutrient supply or by decreasing nutrient demand (Houdijk *et al*., 2005, Normanton *et al*, 2006). However, these results were confounded with increased feed intake. Therefore, effects observed on parasitism and immune responses could have been related to changes in the gut environment (e.g. Petkevicius *et al*, 1999), or increase in energy intake or any other nutrient, as a consequence of increased food intake. The current experiment aimed to account for this uncertainty by testing the hypothesis that parasitism during lactation is sensitive to protein supply and nutrient demand in the absence of changes in food intake. This would then further support the view that nutritional control of gastrointestinal parasitism would be due to improved host immunity.

The current experiment achieved the correct conditions under which this hypothesis could be tested. From the data obtained, it is evident that these feeding treatments affected dam performance i.e., body weight change and litter gain (proxy for milk production). The effects of changes in nutrient demand was to be evidenced by comparing LP9 and LP3 feeding treatments. The aim was to create differences in nutrient demand, so that LP9 rats would be in a nutrient scarce state, while LP3 rats would achieve nutrient abundance. At similar levels of achieved food intake over the 12-day lactation period, LP9 rats lost significantly more weight, compared to LP3 rats. LP9 individual pup weight was significantly lower compared to LP3. Hence, the data support the view that LP9 rats were indeed in a more nutrient scarce state than LP3 rats, although it can not be excluded that nutrient supply to LP3 rats was still scarce, as this group of rats lost body weight during lactation. The effects of changes in protein supply were to be evidenced by comparing HP9 and LP9 feeding treatments. The aim was that HP9 rats would be in a more protein abundant state when compared to LP9 rats. Although there was no significant difference between their weight loss, HP9 pup and litter weight was significantly higher than LP9 pup weight. These results therefore suggest that HP9 rats were in a less protein scarce state compared to LP9 rats. However, as HP9 final individual pup weight was significantly less than LP3 pup weight, it is suggested that HP9 rats could have attained a more protein sufficient state at higher crude protein intake. Houdijk *et al* (2005) concluded that an average CP intake of 5.6g/d is not scarce. Using the data from our current experiment, it was calculated that HP9 average CP intake was 4.27g/d. Therefore, although HP9 rats were in a less protein scarce state compared with LP9 rats, protein supply was likely still scarce.

The discussion will hereafter focus on the number of eggs in the colon, as these changes act as a reflection of how the host has coped with the parasite challenge. As mentioned earlier, the collection of worm burdens was not possible due to the small intestine being used for immunological response analysis. The results from the present study show that at similar food intake, during the post infection period, a clear reduction in the number of eggs in the colon with increased protein supply and reduced nutrient demand was observed on day 10 post secondary infection. At similar feed intake, HP9 rats had a significantly lower number of eggs compared to LP9 rats. In order for the diets to have the same GE content, it was necessary to iso-energetically exchange casein for carbohydrates/oil, which resulted in the diets slightly differing in their ME content. However, this was less than 3%, and many studies have shown that expression of immunity to gastrointestinal parasites is not sensitive to such moderate changes in energy supply (Brown *et al*, 1991, Donaldson *et al*, 1998). As well as differing slightly in ME content, the differences in protein content resulted in the low protein diet containing more fat. However, effects of fat intake on the immune system are usually related to the intake of n-3 rather than n-6 fatty acids (Yaqoob, 2004) of the maize oil used in the present experiment. Therefore, we suggest that the significant differences seen in the number of eggs present in the colon between the treatment groups was likely due to an increased intake of protein.

The observed reduction in egg count from LP9 rats to LP3 rats at a similar food intake points toward an improvement of host resistance in the absence of direct

changes in gut environment. Therefore, these results support the view that the nutritionally induced reduction in the number of eggs present in the colon in our experiment was achieved through the effects of protein availability on expression of immunity.

Through the measurement of immunoglobulins, specific inflammatory cells and rat mast cell protease (RMCPII), we aimed to ascertain some of the possible underlying immune responses that may be associated with nutritionally improved resistance to *N. brasiliensis* during lactation. In general, effector mechanisms against GI nematodes involve antigen-specific T cell responses which induce antibody response and inflammatory changes, with the release of various chemical mediators, which ultimately lead to the expulsion of the worms (Befus, 1995). It is known that primary intestinal nematode infections are typically accompanied by elevated IgE, IgA and IgG1 antibody isotypes (Onah & Nawa, 2000, Negrao-Correa *et al*, 1999). However, there is little evidence to indicate whether these antibodies represent a principal effector mechanism in resistance and worm expulsion for lactating animals experiencing a breakdown of immunity. The current experiment uses a secondary infection in order to investigate the nutritional effects on parasitism during a breakdown of immunity. As very little is known on the effector mechanisms responsible for the expulsion of worms during a secondary infection, our results included the analysis of these specific antibodies, as well as $IgG2_a$. The results showed that there was a trend for LP9 rats to have a lower level of each antibody isotope class when compared to HP9 and LP3 rats. The trend was the mirror image of the significant effects of treatment on the number of eggs in the colon. Other

studies have suggested that the location of the parasite in the intestinal mucosa may be critical in determining whether or not an intestinal antibody response occurs (Negrao-Correa *et al*, 1999). It is known that during a *N. brasiliensis* infection, the worms are able to migrate along the gastrointestinal tract (Wells & McHugh, 1983). As our samples were only taken at one time point, 10 days post infection, this may explain why we were unable to detect any strong differences between treatment groups. Future work will expand upon these preliminary results by including samples taken at various time points during the secondary infection.

Th2 type responses associated with gastrointestinal nematode infections also include specific inflammatory cells. The role of mucosal mast cells as important effector cells in the expulsion of a *N. brasiliensis* infection has been well documented for primary infections (Befus & Bienenstock, 1979), and it has also been suggested that they may play a role during a secondary infection (Katona *et al*, 1988). However, more recent studies have suggested that the final phase of immune expulsion of *N. brasiliensis* in primary infections is mast cell independent (Nawa *et al*, 1994). Infection of mast cell-deficient W/Wv mice have failed to prevent expulsion of *N. brasiliensis* (Madden *et al*, 1991). Our results from the current experiment support this view as there were no significant differences in the number of mast cells present between the varying degrees of parasitism observed in our rats. In contrast, it has recently been suggested that mast cells may indeed have some effects that favour the fecundity of *N. brasiliensis*. This view is supported by experiments where faecal egg output in *N. brasiliensis* infected Ws/Ws mast cell-deficient rats is reduced when compared with mast cell-sufficient littermates (Arizono *et al*, 1993) and, suppression

of MMC hyperplasia in rats infected with *N. brasiliensis* also significantly reduces faecal egg output by the parasites (Newland *et al,* 1995).

There is still much uncertainty regarding the degree of nutritional sensitivity of the mast cell response to *N. brasiliensis* infection. Cummins *et al* (1987) indicated that dietary protein deficiency in growing rats resulted in delaying the expulsion of *N. brasiliensis* from the gastrointestinal tract and that this was associated with a reduction in the number of mucosal mast cells compared to protein-replete controls. The current experiment compared the number of mucosal mast cells and eosinophils in the different treatment groups. It has been shown that the activity of the latter can be influenced by the former (Capron *et al*. 1978). Our analysis showed that increasing protein supply and or reducing nutrient demand had no effect on either parameter. These observations are consistent with results from our previous work, where a reduction in nutrient demand was associated with a reduction in worm burden, but no significant differences were seen for the level of mucosal mast cells, or eosinophils (Normanton *et al*, 2006). Associated with the number of mucosal mast cells is the level of RMCP II present in the GI tract. RMCP II is a mediator, which is released when the mast cells are activated, and the detection of this mediator is very sensitive. Our current experiment included the analysis of RMCP II, as our previous experiment showed no significant treatment effects on the number of mucosal mast cells present in the gastrointestinal tract. An increase in RMCP II could indicate increased MMC activity, as upon antigenic stimulation through IgE, MMC release their contents, including RMCP II (Huntley, 1992). Our result indicate that although

there was a trend for LP9 rats to have a ~10% lower level of RMCP II compared with LP3 rats, this trend failed to reach statistical significance.

In addition to analysing mast cell response, we also aimed to analyse the number of globule leukocytes present in the small intestine. After appropriate antigenic stimulation, mucosal mast cells release their contents and gradually develop into transitional cells and then into globule leukocytes (Huntley, 1992). Connan (1973) found globule leukocytes from samples obtained after more than ten days of a single primary infection with *N. brasiliensis*. However, globule leukocytes were absent in all of the mucosal tissues collected. This is in contrast to previous findings where the presence of an effect of protein supplementation on globule leukocytes has been consistently observed in other parasitised mammals (Houdijk *et al*. 2005b). However, in these studies a gradual, trickle infection has been used, in comparison to our short, single period of infection. This might explain why globule leukocytes were absent in the mucosal samples, as well as perhaps the timing of the sample used.

The current experiment found no significant differences between cellular activities for the different treatment groups. Goblet cell involvement during a secondary infection with parasites, remains to be elucidated. Increased numbers of goblet cells and qualitative changes in mucus secretion have been reported following a primary infection with a number of nematode parasites (Mckenzie *et al*, 1998), and it has been proposed that mucin proteins mediate this response by enveloping the parasites and/or interrupting adhesion (Nawa *et al*, 1994). Therefore, future work intends to

investigate the role of goblet cells and nematode expulsion during a secondary infection.

In conclusion, the results of this experiment support the view that the extent of *N. brasiliensis* infection during the periparturient period is sensitive to changes in protein supply and nutrient demand. Since these effects were observed in the absence of differences in food intake *per se*, they support the view that nutritional control of resistance to parasites during lactation is mediated through host immunity. The underlying mechanisms of the nutritional basis of relaxation in immunity during the periparturient period still remain to be elucidated, but our preliminary immunological results provide a good basis for the rodent model to be used to increase our understanding of the mechanisms involved.

Chapter Four

The effect of increments in protein supply on lactational performance and immunity to *Nippostrongylus brasiliensis* in lactating rats

4.1 Abstract

It has been shown that increasing protein supply at times of protein scarcity reduces the number of *Nippostrongylus brasiliensis* in lactating rats. However, it is not known how gradual increments of scarce metabolisable protein may affect milk production and the breakdown of immunity. The objective of the current experiment was to assess the effects of a gradual increase in protein supply on resistance and immune responses to *N. brasiliensis* in lactating rats. 48 rats were given a single dose of 1600 *N. brasiliensis* larvae prior to mating (primary infection), and re-infected with the same dose on day 2 of lactation. During lactation, rats were offered one of 6 levels of dietary metabolisable protein, ranging from 1.75 to 6.75 g CP/day. Litter size was standardised to 9 pups by day 2 of lactation. Rats were slaughtered on day 10 post secondary infection to assess the concentration of worm burdens and nematode eggs in colon contents. Inflammatory cell concentrations (mucosal mast cells, eosinophils and globule leukocytes), local antibodies (IgA, IgE, IgG_1 and IgG_{2a}) and rat mast cell proteases (RMCP-II) were measured. Feeding treatments affected quadratically the number of worms and eggs present in the colon. They decreased as the protein contents of the diets increased. Feeding treatments did not affect the levels of IgA, IgE, IgG_1, IgG2a, eosinophils, mucosal mast cells or RMCP II levels on day 10 of secondary infection. The effects of increasing metabolisable protein supply on milk production and immune functions, suggest that scarce metabolisable protein is allocated towards milk production as well as immunity.

4.2. Introduction

During the periparturient period, the expression of acquired immunity to a wide range of pathogens usually breaks down (Houdijk *et al*, 2001); for gastrointestinal parasites, this is characterised by an increased worm burden and elevated excretion of nematode eggs with the faeces into the environment. A recently proposed nutrient partitioning framework puts forward a nutritional basis for this periparturient breakdown in immunity (Coop & Kyriazakis, 1999). This nutrient-partitioning framework suggests that the allocation of scarce nutrients to bodily functions associated with parasite control may have a lower (partial) priority than those associated with reproductive effort. As a consequence, at times of nutrient scarcity, which often occurs during the periparturient period because nutrient requirements are considerably elevated relative to non-reproducing hosts (Houdijk *et al*, 2001), hosts would experience a breakdown of immunity to parasites.

It has been shown that lactating rats exhibit a breakdown of immunity to *Nippostrongylus brasiliensis* (Houdijk *et al*, 2003*a*), and further studies with this model have shown that the associated elevated worm burdens are sensitive to changes in the degree of nutrient scarcity. Houdijk *et al* (2005*a*) and Normanton *et al* (2006) have shown that feeding high protein foods during lactation results in a reduced worm burden, when compared to lactating rats receiving low protein foods. In addition, Normanton *et al* (2007) showed that worm burdens also reduce with reduced nutrient requirements that result from a reduction in the reproductive effort.

Although these studies support the view that the breakdown of immunity to *N. brasiliensis* has a nutritional basis, the underlying immune responses that may be associated with this nutritional sensitivity still need to be identified. It is known that *N. brasiliensis* is a potent Th2 inducer, which gives rise to specific effector mechanisms, such as isotype-specific humoral responses and non-specific inflammatory responses, that lead to final worm expulsion (Maizels *et al*, 2004). Intestinal nematode infections are typically accompanied by elevated concentrations of IgE, IgA and IgG_1 antibody isotypes (Onah & Nawa, 2000). As well as these humoral responses, several studies have demonstrated that gastrointestinal nematode infections are invariably accompanied by inflammatory cell responses, such as an increased concentration of mucosal mast cells, rat mast cell protease (RMCP II), eosinophils, goblet cells and globule leukocytes (Miller, 1996, Shin *et al*, 1997, Dent *et al*, 1999).

The objective of the current experiment was to test the effects of gradually increasing protein supply from scarce to more than adequate on lactational performance, resistance and immune responses to *N. brasiliensis* in lactating rats. It was hypothesised that the initial increments of scarce protein will be firstly allocated to lactational performance, resulting in an increased litter weight gain (proxy for milk production) without affecting the extent of breakdown of immunity to *N. brasiliensis*. Subsequent increments would not affect litter weight gain, as requirements for the prioritised milk production would have been met, but would reduce the extent of breakdown of immunity to *N. brasiliensis* since the rat can now allocate more protein to expression of immunity. To test this hypothesis, we have used six levels of protein

supply, ranging from scarce to more than adequate which would be needed to assess the expected different degrees of penalty for both reproductive functions and immune functions. In addition, previous work with this model have used only one level of scarce protein supply, and under these conditions, different degree of penalties on reproductive effort and immunity can not be assessed (Houdijk *et al*, 2003*b*). Our previous studies (Normanton *et al*, 2006, 2007) found that reducing nutrient scarcity tended to affect local humoral responses in the gut with little effect on inflammatory responses, even though worm burdens were significantly reduced. Here, we assess the same parameters, as it can not be excluded that nutritional sensitivity of these responses may depend on the levels of scarce or adequate protein nutrition used.

4.3. Materials and Methods

4.3.1. Animals and housing

Forty-eight female Sprague-Dawley rats (Harlan Ltd, Oxfordshire, UK) were housed in a room where ambient temperature was maintained at 21°C, relative humidity ranged from 45 to 65%, and artificial lighting was provided between 08.00-18.00 hours. Rats were housed in solid-bottomed cages with fresh sawdust being provided weekly and a handful of shredded plastic bubble wrapping for nesting material from 3 days before expected parturition until the end of the experiment. Wire-bottomed cages were used during mating and for faeces collection during the primary infection as described previously (Houdijk *et al*. 2003*a*). For mating, female rats were placed

with a proven male breeder and mating was confirmed through the presence of a vaginal plug.

4.3.2. Foods and experimental design

All rats were given *ad libitum* access to standard rat chow until mating was confirmed. Mated rats were then given *ad libitum* access to a high crude protein (CP) food (210g CP per kg DM) for 10 days followed by a low CP food (60g CP/kg DM) until parturition. This feeding protocol was used to reduce body protein reserves during the second half of gestation in order to maximize the degree of protein scarcity during lactation when rats are on low protein foods (Pine *et al*. 1994; Houdijk *et al*. 2005*a*).

Parturition was considered as day 0 and from then until day 12 of lactation, rats were allocated to one of six feeding treatments A, B, C, D, E and F, which were designed to supply on average 1.75, 2.75, 3.75. 4.75, 5.75 or 6.75g of CP per day, respectively over the 12 days of lactation. This range was based on feeding treatments used in a previous experiment, where 1.75g of CP per day resulted in protein scarcity and 6.75g of CP per day resulted in protein sufficiency (Houdijk *et al*, 2005*a*). Using the results from Houdijk *et al,* (2005*a*), it was calculated that the CP supply will change from scarce to adequate as it increases from 4.75g to 5.75g per day. To avoid the previously observed changes in food intake associated with manipulation of dietary CP contents (Houdijk *et al*, 2005*b*) experimental foods were offered in restricted amounts. The latter was based on a percentage of their parturition body weight in

order to achieve the required level of CP intake: A at 6%, B at 6.3%, C at 6.7%, D at 7.0%, E at 7.4% and F at 7.7%. The diets were formulated to provide, at these levels of intake, the same amount of non-CP dry matter per kg parturition bodyweight. Consequently, in the absence of refusals, the experimental design was expected to result in a linearly increased DM intake between the feeding treatments, which would only be attributed CP. Ingredients and chemical analysis of the experimental foods offered during lactation are shown in Table 1. The litter size of each treatment group was standardised on day 2 of lactation at 9 pups.

Table 1. Composition and chemical analysis of the experimental foods used during lactation.

	Treatment groups					
Ingredients (g/kg fresh matter)	A	B	C	D	E	F
Casein (plus 1% Methionine)	100	150	190	230	270	301
Starch	292	273	262	249	235	228
Sucrose	146	138	132	125	118	114
Corn oil	210	199	189	180	171	160
Vitamins	50	48	45	43	41	39
Minerals	50	48	45	43	41	39
Cornflour	43	41	39	37	35	34
Choline	7.0	6.7	6.3	6.0	5.7	5.5
Lecithin	2.0	1.9	1.8	1.7	1.6	1.6
Alphacel	100	95	90	86	82	78
Analysed chemical composition (g/kg dry matter)						
Dry matter (g/kg fresh)	71.4	80.0	75.0	68.8	65.0	62.5
Gross Energy (MJ/kg DM)*	19.7	19.9	20.1	20.3	20.4	20.6
Crude protein	106	156	191	237	277	311

* Calculated from feed tables

4.3.3. Infection protocol

All rats were infected with *N. brasiliensis* according to a previously established protocol (Houdijk *et al*. 2003*a*). Rats received on day$_{-45}$ (45 days before the realised mean parturition date) a primary infection of 1,600 third-stage infective larvae L_3 of *N. brasiliensis*, which were suspended in 0.5 ml sterile phosphate buffered saline (PBS). A secondary infection of 1,600 L_3 *N. brasiliensis* was administered on day 2 of lactation.

4.3.4. Body weight and food intake

Rats were weighed daily throughout the experiment. Intake was measured daily during gestation and any refusals that did occur during lactation were collected every morning and weighed. The pups were counted and weighed daily from day 0. Foods offered during gestation & lactation were sampled during their preparation for the analysis of DM and crude protein (Kjeldahl-N x 6.25).

4.3.5. Nematode egg counts and worm counts

Seven days post primary infection (day$_{-38}$ relative to parturition) faeces were collected overnight as described previously (Houdijk *et al*. 2003*a*), for the assessment of faecal egg counts (eggs per g of faeces). This was done to provide evidence that a primary infection had established.

All rats were sedated by gradually increasing ambient concentration of CO_2 and humanely killed by CO_2 asphyxiation on day 12 and dissected to collect the large and small intestine. Large intestinal contents were assessed for the number of nematode eggs as per Houdijk *et al* (2003*a*). At slaughter, nematodes were harvested from the small intestine, both as described before (Houdijk *et al*. 2003a). Unlike in previous experiments (Houdijk *et al*, 2005*a*, Normanton *et al*, 2007) worms were not collected from the first 20 cm of the gut, as this was used to collect mucosal scrapings to measure immune responses (see below).

Immune responses

4.3.6. Inflammatory responses

A small 2 cm section of small intestine, 15 cm down from the stomach, was placed in 4% paraformaldehyde for 6 h and then transferred to 70% ethanol. The small intestine sections were embedded in paraffin wax and mounted on slides for histochemical quantification of inflammatory cells. Sections of tissue were stained with toludine blue at pH 0.5 for the assessment of mucosal mast cells counts, whilst globule leukocytes and eosinophils counts were assessed after staining with carbol chromatrope and differentiation on morphological criteria (Huntley *et al*. 1995).

4.3.7. Local antibodies in mucosal scrapings

Mucosa scrapings were obtained by scraping approximately 20cm of the anterior small intestine with a glass microscope slide. Samples were then transferred into a bijoux tube and frozen. For the analysis of antibody and RMCP II, (see below), the samples were thawed and homogenised on ice, using ice-cold PBS, to solubilise the mucosa sample.

An enzyme linked immunosorbent assay (ELISA) method was used for the detection of the antibody isotypes IgG1, IgG2a, IgA and IgE in mucosal tissue. High binding ELISA plates were coated with 10µg/ml of adult *N. brasiliensis* antigen, in 50 µl/well bicarbonate buffer pH9.6 for 1 hour. Plates were then washed three times with PBS/Tween 20/NaCl solution. A standard was used on each plate to account for plate to plate variation. This was made from a small pooled selection of mucosal homogenates which tested positive for antibodies in previous studies. Mucosal homogenates for each rat were used in dilution 1 in 10 in PBS. The mucosal homogenate samples and the standards were added in 50 µl/well PBS and incubated for 1 hour at room temperature, following which plates were washed six times as above. Isotype-specific secondary antibodies were then added (Serotec, UK). All secondary antibodies were diluted in PBS/Tween 80 at a 1/1000 dilution and incubated on the plates for 1h at room temperature. Following washing six times as above, horse radish peroxidase (HRP) conjugated anti-mouse immunoglobulin antibody (Serotec, UK) was added to each well (50µl). This was again incubated for 1 hour. Plates were then washed six times as above, and then the antibody isotypes

could be detected by the addition of 50µl/well of Sigma Fast-OPD and the colour allowed to develop for around 15 minutes before stopping with 2.5 M H_2SO_4. Following colour development, optical density values for each well were read at 492 nm (OD_{492}).

4.3.8. Rat mast cell protease-II

RMCP-II levels were detected and quantified from mucosa by ELISA as described by Miller et al (1983), using a commercially available kit (Moredun Scientific Limited, RMCP II ELISA). Assays were carried out according to manufacturer's instructions. ELISA plates were coated with 2 µg/ml anti-RMCP II monoclonal antibody in bicarbonate buffer pH 9.6 at 4°C overnight. Following six washes with phosphate buffered saline with Tween-20 (PBST) plates were blocked for unspecific protein at 37°C for 30 min with 4% bovine serum albumin in PBST. Plates were washed again three times and standards in series 0.5-12 ng/ml of RMCP II in PBST were added in. Mucosal homogenates were then plated at four dilutions, i.e. 1:10, 1:100, 1:1000, and 1:10,000, and incubated at 37°C for 30 min. Following a further six washes, plates were incubated for 1h with 50µl/well conjugated antibody solution at 37°C. Plates were once again washed six times before addition of 50 µl/well substrate (specific to the RMCP II kit) to allow for the colour formation. Colour formation was allowed to progress for 15 min before stopping with 0.25M H_2SO_4. OD values for each well were then read at 450 nm on a microplate reader and RMCP II levels (ng/ml) calculated by comparison against a plotted standard curve.

4.3.9. Calculations and statistical analysis

The aim was to obtain 7 replicates for each of the six feeding treatments. However, 15 rats did not conceive, 2 rats were euthanised as they developed tumour-like swellings, and 8 rats did not rear the 9 pups required. Therefore, achieved number of replicates for A, B, C, D, E and F were 6, 4, 4, 3, 2 and 4, respectively. It might be argued that the resulting small number of replicates for most feeding treatments would hamper statistical analysis through one-way analysis of variance. However, we were not necessarily interested in differences between specific feeding treatments but mainly in the shape of the dose-response relationship between protein supply on the one hand and lactational performance and immunity to *N. brasiliensis* on the other hand. Therefore, although we would like to have ended up with more replicates, we consider that the obtained data set can still be analysed through exploring orthogonal polynomial statistics (see below).

Due to their skewed nature, parasitological and immunological results were transformed according to log (n+1) to normalize data before statistical analysis. The transformed data are reported as backtransformed means, accompanied by a backtransformed lower and upper limit. These were calculated via 10^a-1 where a = $\mu+0.5\times\sigma^2$ (Johnson *et al*, 1988), with μ, μ - s.e. and μ + s.e. as the mean, lower and upper limit of the transformed data, and σ as their standard deviation. Consequently, backtransformed lower and upper limits characterise an unequally distributed range

around the backtransformed means. Non-transformed data are presented as arithmetic (least-square) mean with standard error (S.E.) or standard error of the difference (S.E.D.)

The main emphasis was on assessing the relationship between feeding treatment and response parameters. This was done through fitting linear and quadratic relationships between feeding treatment on the one hand, and performance, parasitism and immune responses on the other hand. Body weight at parturition was used as a covariate when analysing the effects of treatments on maternal weight during lactation. Although desired, the number of feeding treatments used was considered too low to analyse the data according to a "broken stick" methodology**.** The expectation was to observe linear effects between feeding treatments and achieved DM and CP intake but quadratic effects between feeding treatments and host response. Protein supply was expected to gradually increase from scarce to more than adequate for lactational effort, thus reaching an asymptote at higher levels of CP intake. We expected parasitism to fall and immunological responses to increase only at these levels. Such relationships between CP supply, lactational effort and immunity to *N. brasiliensis* could be described as quadratic. All statistical analyses were performed using Genstat 6 for Windows (release 6.1, 2002; Lawes Agricultural Trust, Rothamsted, Herts., UK) and Minitab 12 (release 12.1, 1998; Minitab Inc.).

4.4. Results

4.4.1. Faecal egg counts during the primary infection and performance until parturition

All rats showed signs of a primary infection, and on day$_{-40}$ the mean faecal egg count was 5,954 (2,949-12,022) eggs per gram. During the first 10 days of gestation, rats grew from 269 (S.E. 6.2) g to 303 (S.E. 4.8) g, with an average DM intake of 20.6 (S.E. 1.2) g/day. From then onwards and until parturition, the pregnant rats continued to grow to a mean weight of 358 (S.E. 10.1) g with an average DM intake of 15.8 (S.E. 0.1) g/day, which dropped to an average of 5.2 (S.E. 0.09) g on the day before parturition.

4.4.2. Food intake, dam and litter body weight during lactation

Diet and time significantly interacted for achieved DM intake (P=0.001) with effects of feeding treatments seen on various days during lactation. On day 3, feeding treatment had a linear effect (P=0.01), but not a quadratic affect (P=0.25) on DM intake. Between days 4 and 10, feeding treatments did not cause a linear response on DM intake (P=0.26-0.86), whilst on day 11 and 12, there was a linear trend (P=0.06). On days 4, 5, 6, 7, 8, 9, 10, 11, and 12, feeding treatments quadratically affected daily DM intake (P=0.03 on all days).

Feeding treatment did not have a linear effect (P=0.98) on mean achieved DM intake, but it did have a quadratic affect (P=0.01). Achieved mean DM intake for treatments A, B, C, D, E and F averaged at 13.7, 19.0, 20.1, 19.55, 13.0, and 15.0 g/day, respectively (S.E.D. 3.6). Consequently, achieved CP intake for treatments A, B, C, D, E and F averaged 1.3, 2.7, 3.6, 4.4, 3.9 and 4.6 g/day, respectively (S.E.D. 0.72; P=0.02), as shown in figure 1.

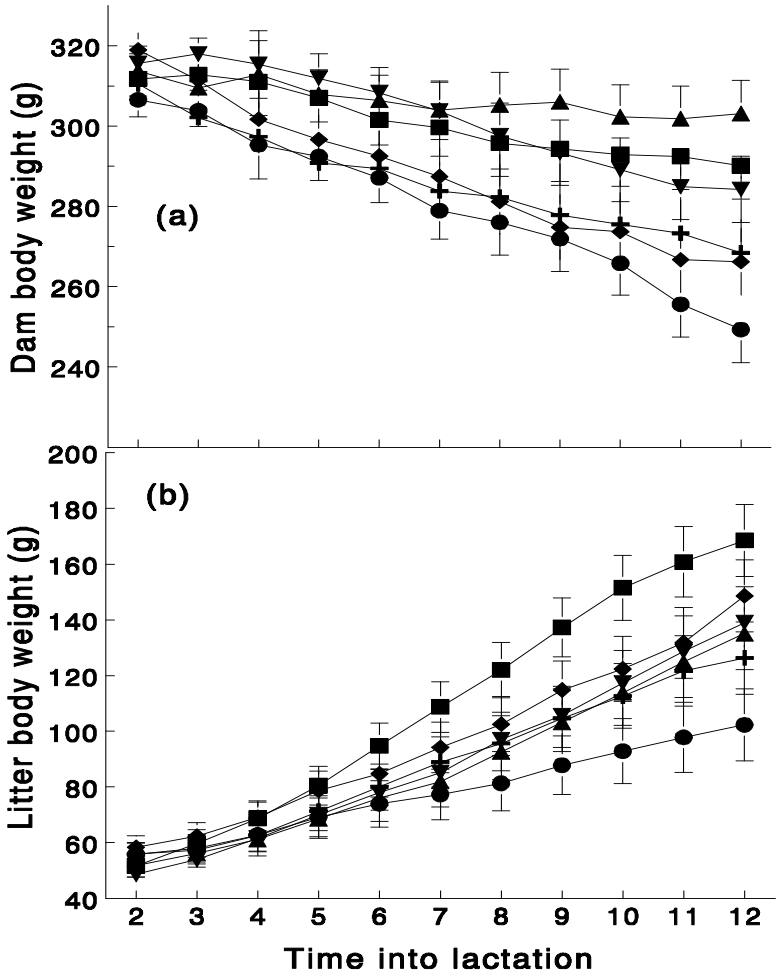

Figure 1. Least square mean (with S.E.) dam body weight (a) and litter body weight (b) of lactating rats, restrictedly offered foods that were calculated to supply a mean of 1.75 (●), 2.75 (▼), 3.75 (▲), 4.75 (■), 5.75 (✚) and 6.75 (♦) g CP/day.

4.4.3 Dam performance

Figure 1a shows dam body weight from day 2 of lactation. At parturition, mean dam body weight was 307.9 (S.E. 8.9) g, which did not differ between feeding treatments (P=0.28). Feeding treatment and time did not significantly interact for dam body weight during lactation (P=0.32). However, feeding treatment quadratically affected mean body weight loss over the 12 day lactation period (P=0.004), with body weight loss for treatments A, B, C, D, E and F averaging at 46, 20, 12, 26, 39 and 39 g, respectively (S.E.D. 17.3g/day). There was no linear effect of feeding treatment on mean body weight loss (P=0.95).

4.4.4. Litter performance

Mean litter weight from day 2 of lactation is shown in Figure 1b. At parturition, mean litter weight was 57 (S.E. 4.8) g, which did not differ between feeding treatments (P=0.59). However, feeding treatment and time significantly interacted for mean litter weight (P=0.004), which was reflected as divergent growth curves over time. On day 12, mean litter weight averaged at 102, 139, 135, 169, 126 and 149g for feeding treatment A, B, C, D, E and F, respectively (S.E.D. 22.7: P=0.003). There was a significant quadratic response (P=0.003), but not linear (P=0.29), of feeding treatment on mean litter gain. Feeding treatment A rats achieved the lowest mean litter gain. An increase in mean litter weight was then seen up to and including the D rats, which achieved the highest mean litter gain. As intended protein supply increased for treatments E and F, but litter gain was lower.

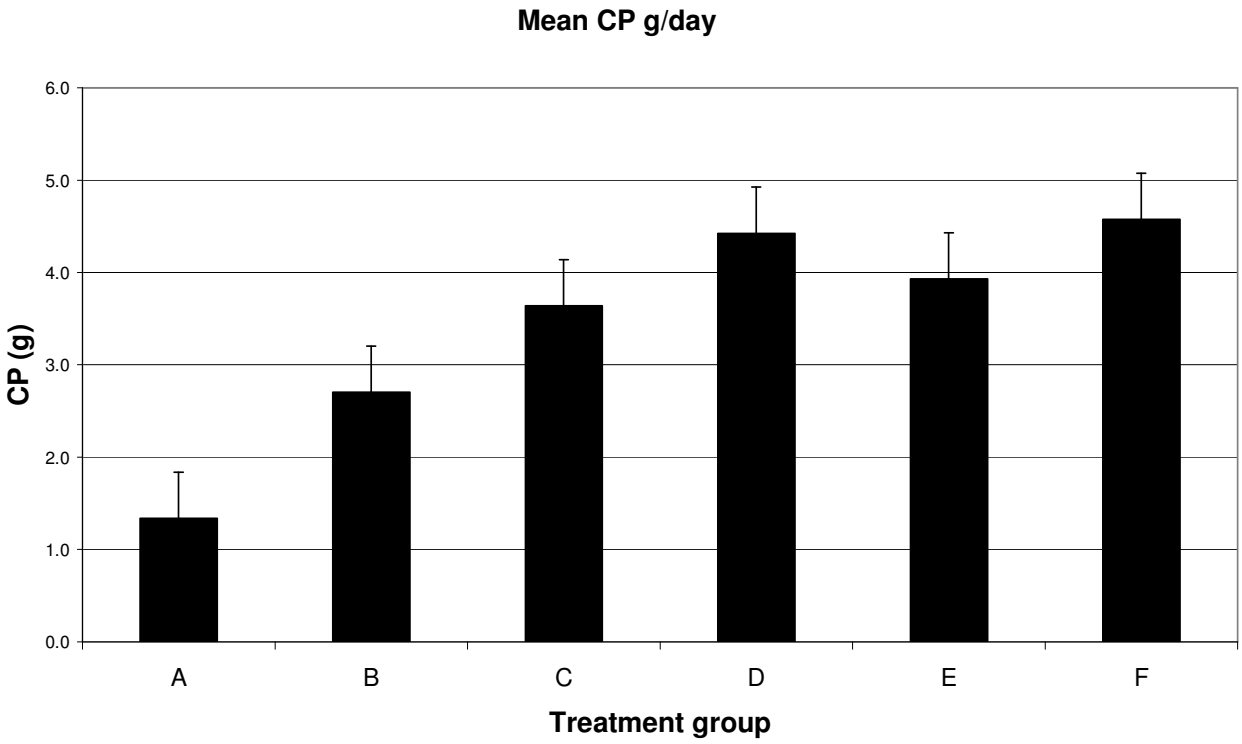

Figure 2. Mean crude protein intake of lactating rats, restrictedly offered foods that were calculated to supply a mean of 1.75 (A), 2.75 (B), 3.75 (C), 4.75 (D), 5.75 (E) and 6.75 (F) g CP/day.

4.4.5. Colon egg count and worm burden

The effect of feeding treatments on the backtransformed mean number of eggs in the colon and worm burden are shown in Figure 3. Feeding treatment quadratically affected the number of eggs present in the colon (P=0.002). There was no linear affect (P=0.73). The number of eggs gradually decreased as the protein contents of the diets increased. Feeding treatment A rats had the highest number of eggs, and this number was subsequently reduced with D, E and F rats having similar low number of eggs present in the colon. These effects were mirrored in the worm burden taken

from the small intestine, with a non significant linear effect (P=0.37), and a quadratic effect of feeding treatment on total worm burden (P=0.06). As with the number of eggs in colon, A rats had the highest worm burden, and this decreased as the protein contents of the diet increased but did not differ between groups B, C, D, E and F.

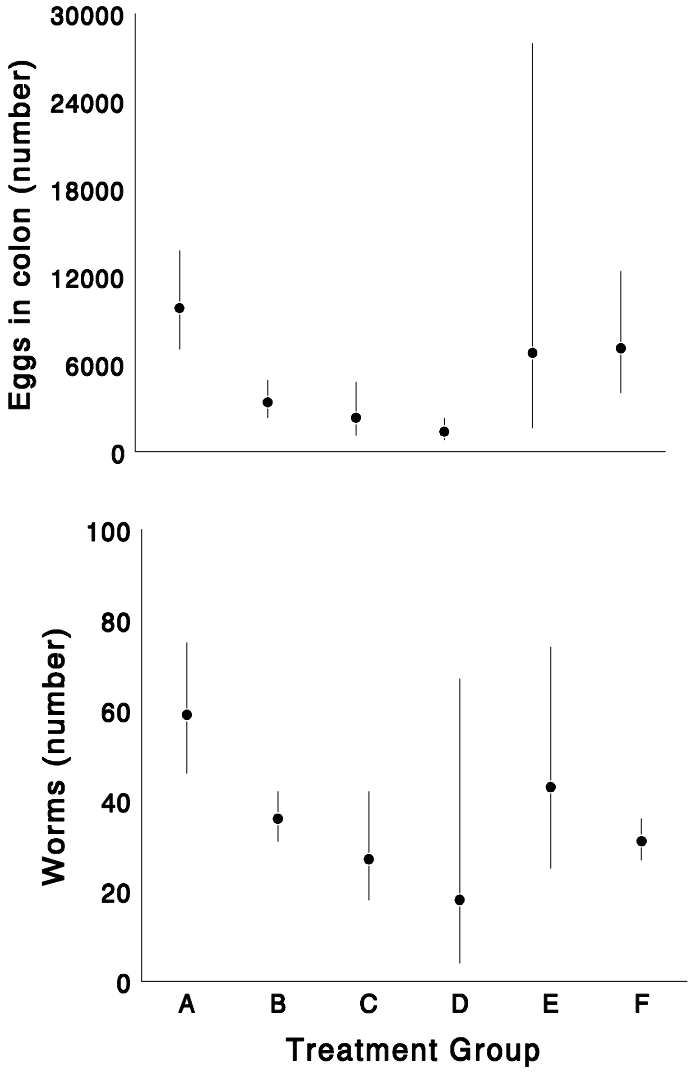

Figure 3. Mean backtransformed (with backtransformed standard errors) number of eggs in colon contents and worm burden of lactating rats, restrictedly offered foods that were calculated to supply a mean of 1.75 (A), 2.75 (B), 3.75 (C), 4.75 (D), 5.75 (E) and 6.75 (F) g CP/day.

4.4.6. Immunological analysis

Feeding treatment did not have a linear or quadratic effect on the concentration of mucosal mast cells (P=0.56, 0.78) or eosinophils (P=0.68, 0.71) present in the small intestine. Overall, the mean concentration of mucosal mast cells was 44 per mm^2 (36-55), and the mean number of eosinophils was 123 per mm^2 (114-133). Globule leukocytes were not observed in all tested sections. In addition, as shown in Figure 4, feeding treatments did not have a linear or quadratic effect on the OD_{492} of IgA (P=0.31, 0.23), IgE (P=0.39, 0.10), IgG_1 (P=0.66, 0.51) or IgG_{2a} (P=0.91, 0.38) present in the mucosa. Feeding treatment also did not have a linear or quadratic affect on the concentration of RMCP II present in the mucosa (P=0.91, 0.53). Overall, the mean concentration of RMCP II was 6.19 ng/ml (5.57-6.87).

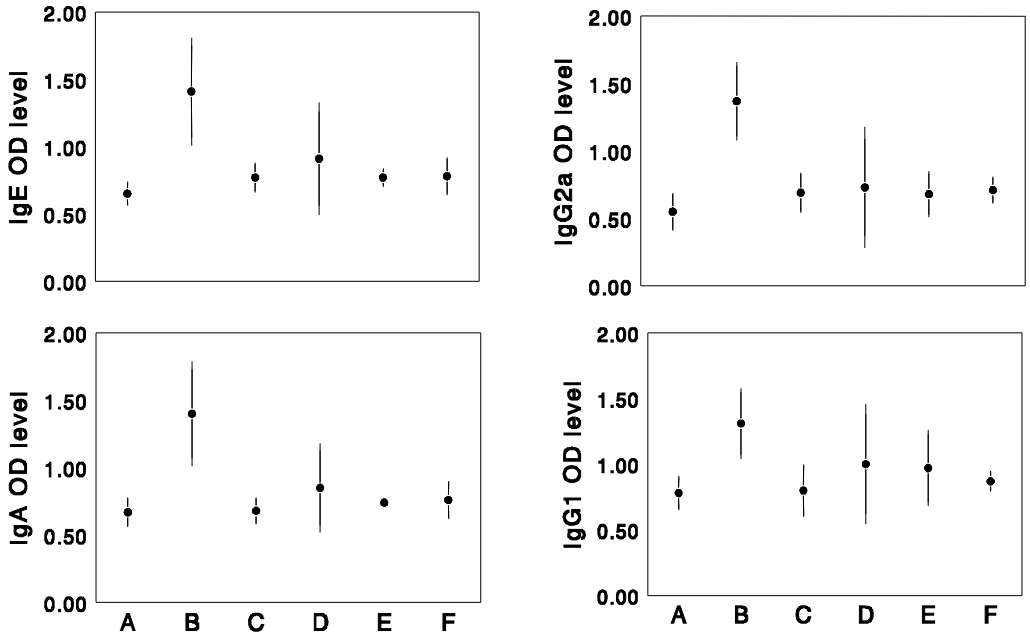

Figure 4. Mean backtransformed (with backtransformed standard errors) OD_{492} of mucosal IgA, IgE, IgG1 and $IgG2_a$ from lactating rats, restrictedly offered foods that were calculated to supply a mean of 1.75 (A), 2.75 (B), 3.75 (C), 4.75 (D), 5.75 (E) and 6.75 (F) g CP/day.

4.5. Discussion

Previous experiments using this rodent model have shown the lactating rat may experience a breakdown of immunity to *N. brasiliensis*. This is evidenced by an increased gastrointestinal nematode burden and a faecal egg count (Houdijk *et al*, 2003*a*). It has also been shown that during this period, a significant reduction in gastrointestinal parasitism can be achieved by either increasing dietary protein content (Houdijk *et al*, 2005*a*, Normanton *et al*, 2006) and decreasing nutrient demand (Normanton *et al*, 2006, 2007). However, these previous experiments have used only one level of scarce and one of adequate protein nutrition, and consequently, they provide only limited insight in the relationship between protein supply and immunity to parasites, and no insight in the degree of penalty of protein scarcity on lactational performance and resistance to *N. brasiliensis*. The current experiment aimed to elucidate this relationship through focusing on effects of gradually increasing protein supply on lactational performance and immunity to *N. brasiliensis*. It was hypothesised that scarce metabolisable protein will be firstly allocated to lactational performance, and only when the rat reaches a protein abundant state will there be an improved immunity, evidence by a reduction in nematode egg count and worm burden.

We first have to judge whether the current experiment achieved the correct conditions under which this hypothesis could be tested. Based on our previous work (Houdijk *et al* 2005*a*, Normanton *et al* 2006), the expectation was that feeding treatments A through D would result in protein scarcity, and that E and F would result in an abundant protein supply. Therefore, A rats would have the greatest

degree of protein scarcity, and as protein supply gradually increased over treatments B, C and D, rats would increase their lactational performance to a maximum, to be achieved in E and F rats. Because the latter two groups would only then be in a protein adequate state, the expectation was that only E and F rats would also show improved immunity to *N. brasiliensis*. From the data obtained, it is evident that the feeding treatments indeed affected dam performance i.e., body weight change and litter gain (proxy for milk production) but in a different manner than expected. The results show that the feeding treatments had a quadratic effect on dam body weight loss and mean litter gain, but there were no indications that the E and F rats' lactational performance had reached the expected asymptote. Feeding treatment A rats lost the most amount of body weight and had the lowest mean litter gain, whilst C and D rats lost the least amount of dam body weight and had the highest mean litter gain. These results suggest that the D rats were more than likely in a protein abundant state under the conditions of the experiment. However, BW loss and litter gain of E and F rats were not similar as expected but were lower than that of C and D rats, indicating that these feeding treatments had led to a lower degree of protein nutrition than expected. The experimental design was expected to result in a linear effect of feeding treatment on achieved DM intake. However, the observed relationship between feeding treatment and DM feed intake was quadratic, and results show that DM intake of especially E and F rats was lower than expected. Consequently, their mean CP intake per day was the same as for the D rats. These results would suggest that E and F may have avoided excess protein intake. It remains unclear why this occurred, as similar 30% CP foods led to higher achieved DM intake when used *ad libitum* (Houdijk *et al*, 2005*a*) or restrictedly (Normanton

et al 2006). However, the previously used 30% CP foods differed in composition of non-CP dry matter; the current high protein foods had a lower proportion of oil and a higher proportion of starch and sugar. The achieved non-CP GE intake for A, B, C, D, E and F rats was 0.20, 0.25, 0.24, 0.22, 0.15 and 0.14 MJ/day, respectively. Hence, this may suggest that E and F rats did not receive enough non-CP energy from their specific diets to metabolise the excess protein supplied. The reduced food intake may therefore be a result of a trade-off between ingesting more protein and inability to deal with excess protein.

Gradually increasing protein supply also caused a quadratic response in reducing the number of worms and eggs in the colon on day 10 post secondary infection. When re-analysing the results with D, E and F rats grouped together, which may be justified from the fact that they had achieved similar CP intake, we still observed a strong trend for a quadratic relationship between feeding treatment and the number of worms present in the gut (P=0.06). However, in contrast to expectation, worm burden and the number of nematode eggs present in the colon concurrently reduced with increased milk production. This is evidenced by the increase in mean litter gain and the decrease in worm burden and nematode eggs when comparing treatment groups A, B, C vs D+E+F. Therefore, our results imply that scarce metabolisable protein is partitioned to milk production as well as expression of immunity. A study carried out by Houdijk *et al*, (2003*b*) also observed a similar relationship between metabolisable protein supply, milk production and nematode egg excretion, but not final worm burden in sheep. However, the relationship between metabolisable protein supply and worm burden may have been different, and more in agreement

with our observed results, had the worm burdens been assessed at times of maximum differences in FEC.

Although our data relating to the number of nematode eggs in the colon are in agreement with that of Houdijk *et al*, (2003*b*), and together with our worm burden results they suggest that scarce metabolisable protein is not prioritised to milk production, it is important to remember that our achieved number of replicates for each treatment are not ideal. In addition, unlike in our previous experiments, the difference in days between primary infection date and parturition was an additional 10 days. However, our non pregnant rats which were infected using the same protocol had no worms and no signs of eggs in their colon contents, suggesting that the difference in time had no effect on their ability to mount an effective immune response. Therefore, to fully understand the relationship between allocation of scarce metabolisable protein supply, milk production and immune expression, this experiment would need to be repeated with more replicates and using the same diets and also the diet formula used in previous experiments (Normanton *et al*, 2007 & 2006) in order to achieve no food refusals.

Although this current experiment used diets which were formulated differently to our previous experiments, in order to ensure that all feeding treatments received the same amount of non CP energy but varied in metabolisable protein content, our results for the number of eggs in the colon for A rats was comparable to that of the low protein rats in Normanton *et al*, (2007 & 2006). However, the worm burdens observed in our current experiment are nearly half as many as were present in the

low protein rats in Normanton *et al*, (2007). The differences observed are more than likely associated with the fact that we used a smaller proportion of the gut, and it was the posterior section as we used the anterior section to collect mucosal scrapings. This suggests that a substantial number of worms are more than likely present in the first 20cm of the small intestine.

The aim of the current experiment was also to investigate effects of gradually increasing protein supply on immune responses during the breakdown of immunity. As very little is known about the effector mechanisms responsible for the expulsion of worms during a secondary infection of *N. brasiliensis*, especially during lactation, we aimed to ascertain some of the possible underlying immune responses that may be associated with nutritionally improved resistance to *N. brasiliensis* during lactation. This was achieved through the measurement of local immunoglobulins, specific local inflammatory cells and RMCP-II on day 10 post secondary infection. In general, effector mechanisms against GI nematodes involve antigen-specific T cell responses which induce antibody response and inflammatory changes, with the release of various chemical mediators, which ultimately lead to the expulsion of the worms (Befus, 1995). The results showed that increasing protein supply had no effects on the level of immunoglobulins present in the mucosa. Again, these results may have been affected by the reduced feed intake of especially the E and F rats, or it may have been due to the single time point sampling. Other studies have suggested that the location of the parasite in the intestinal mucosa may be critical in determining whether or not an intestinal antibody response occurs (Negrao-Correa *et al*, 1999). It is known that during a *N. brasiliensis* infection, the worms are able to

migrate along the gastrointestinal tract (Wells & McHugh, 1983). As our samples were only taken at one time point, i.e. 10 days post infection, the worms might have already migrated past the critical location for a response to be seen, and this may explain as to why we were unable to detect any strong differences between feeding treatments. Future work will expand upon these preliminary results by including samples taken at various time points during the secondary infection, and could include sampling at different sites of the gut to account for the mentioned migration.

Th2 type responses associated with gastrointestinal nematode infections also include specific inflammatory cells. It has been suggested that mucosal mast cells may play a role during a secondary infection (Katona *et al*, 1988). However, more recent studies have suggested that the final phase of immune expulsion of *N. brasiliensis* in primary infections is mast cell independent (Nawa *et al*, 1994). Our results from the current experiment support this view as there were no apparent relationships between the concentration of mast cells and the varying degrees of parasitism observed. It has been shown that the activity of eosinophils can be influenced by mucosal mast cells (Capron *et al*. 1978). Our analysis showed that feeding treatments had no affect on the number of eosinophils present in the small intestine. These observations are comparable and consistent with results from our previous work, where a reduction in nutrient demand was associated with a reduction in worm burden, but no significant differences were seen for the level of mucosal mast cells, or eosinophils (Normanton *et al*, 2006, 2007). Associated with the number of mucosal mast cells is the level of RMCP II present in the GI tract. RMCP II is a mediator, which is released when the mast cells are activated, and the detection of this mediator is very sensitive. Our

results showed that feeding treatment had no significant effect on the concentration of RMCP II present in the gut. However, it can not be excluded that differences in mast cell activity, as indicated by differences in RMCP-II concentrations, in antibody OD_{492} or inflammatory cell concentration may have occurred prior to day 10 post secondary infection.

In addition to analysing mast cell response, we also aimed to analyse the number of globule leukocytes present in the small intestine. After appropriate antigenic stimulation, mucosal mast cells release their contents and gradually develop into transitional cells and then into globule leukocytes (Huntley, 1992). Globule leukocytes have been observed following a primary infection with *N. brasiliensis* (Connan, 1973; Miller, 1996) but whether such responses occur following a secondary infection is not known. In the current study, globule leukocytes were not observed in any of the mucosal tissues collected. Although this seems in contrast to studies with other parasitized mammals, where the presence of an effect of protein supplementation on globule leukocytes has been consistently observed (Houdijk *et al*. 2005*b*), it can not be excluded that mast cell maturation was not (yet) complete at time of sampling. In support of this view, Connan indeed observed globule leukocytes following a primary infection with *N. brasiliensis* from day 10 post infection onwards (Connan, 1973).

In conclusion, the results of this experiment show that during a breakdown of immunity, the lactating rat will partition scarce protein not only to her reproductive effort, but also to her immune response (as reflected in changes in resistance to *N. brasiliensis*). However, the results did not support the view that scarce nutrient

allocation to lactational effort is significantly prioritised over expression of immunity to parasites. The nutritional sensitivity of underlying immune responses that may be responsible, at least in part, for the observed nutritionally improved resistance still remain to be elucidated. The latter may benefit from assessing effects of host (protein) nutrition on immune responses over time, as it can not be excluded that nutritional sensitivity of immune responses is temporal.

Chapter Five

Effects of protein supply on expression of immunity to *Nippostrongylus brasiliensis* at different time points during lactation in rats

5.1 Abstract

Increasing protein supply at times of protein scarcity improves resistance to *N. brasiliensis,* but it is not know whether there is a temporal effect on the local immune responses. This was addressed in a lactating rat model, as lactating rats show a breakdown to the intestinal nematode *N. brasiliensis.* Therefore, the objective of the current experiment was to assess temporal effects of increased protein supply on resistance and immune responses to *N. brasiliensis* in lactating rats. 48 rats were given a single dose of 1600 *N. brasiliensis* larvae prior to mating (primary infection), and re-infected with the same dose on day 2 of lactation. During lactation, rats received a low protein diet (LP, 100g CP/kg DM) or a high protein diet (HP, 300g CP/kg DM). Rats were fed restrictively at 7.5% of their parturition body weight. Litter size was standardised to 9 pups by day 2 of lactation. On day 5, 10, and 15 post secondary infection the concentration of nematode eggs in colon contents was assessed. Mucosal scrapings were taken to assess local antibodies (IgA, IgE, IgG_1 and IgG_{2a}) and rat mast cell proteases (RMCP-II). Diet and time did not significantly interact for colon egg count and for RMCP-II. HP rats tended to have lower colon egg count and had significantly lower concentrations of RMCP-II than LP rats, with effects on RMCP-II. There was a trend for diet and time to interact for IgA, IgE and IgG_{2a} levels. The results support the view that increasing protein supply at times of protein scarcity improves periparturient resistance to *N. brasiliensis*.

5.2. Introduction

The periparturient parasitized animal can be a major source of infection for their parasite-naïve offspring, as expression of acquired immunity to parasites usually breaks down (Houdijk *et al*, 2005b). For gastrointestinal nematodes this is characterised by an increased worm burden and elevated excretion of nematode eggs into the environment. The lactation phase of reproduction in mammals demands maximal nutritional resources (Hudson & White, 1985), and there is increasing evidence to support the view that immune defences are compromised by limiting access to resources (Lochmiller *et al*, 1993). It has been proposed that at times of resource scarcity, a reproducing mammal prioritises the degree to which it allocates the scarce resource available to various body functions (Coop & Kyriazakis, 1999). This nutrient-partitioning hypothesis suggests that the allocation of scarce resources to the functions associated with parasite control have a lower partial priority than those associated with the reproductive effort. As a consequence, at times of nutrient scarcity, which often occurs during the periparturient period, hosts would experience a breakdown of immunity to parasites.

The possible nutritional basis of the periparturient breakdown of immunity to parasites has been addressed in a rat model, as it has been demonstrated that lactating rats exhibit a breakdown of immunity to *Nippostrongylus brasiliensis* (Houdijk *et al*, 2003). It has also been shown that at times of protein scarcity, increasing protein supply and reducing nutrient demand improved resistance to *N. brasiliensis* (Houdijk *et al*, 2005; Normanton *et al*, 2006a). Feeding high protein foods *ad libitum* to

lactating rats results in a reduced worm burden, compared to feeding low protein foods (Houdijk *et al* 2005a), whilst overcoming nutrient scarcity through reducing litter size has resulted in reduced worm burdens in lactating rats offered *ad libitum* a low protein food (Normanton *et al*, 2006a).

Although these studies support the view that the breakdown of immunity to *N. brasiliensis* during lactation has a nutritional basis, the underlying immune responses that may be associated with this nutritionally improved resistance still need to be identified. It has been proposed that a two-step mechanism for worm expulsion exists; specific antibodies cause the damage of worms and subsequently a lymphocyte-mediated non-specific inflammatory response causes the expulsion (Ishiwata *et al*, 2002). One of the possible inflammatory responses could be an increase in goblet cell numbers. Mucins synthesized and secreted by goblet cells in the small intestine form a protective mucus blanket overlying the epithelial surface, and a rise in the number of goblet cells present in the small intestine has been found around the time of immune-mediated expulsion of *N. brasiliensis* (Nawa & Korenaga, 1987). As mentioned earlier, it is also known that intestinal nematode infections are typically accompanied by elevated IgE, IgA and IgG_1 antibody isotypes (Onah & Nawa, 2000), although there is very little consistent and convincing data to indicate these are principal effector mechanisms in resistance to intestinal nematodes. In our previous experiment (Normanton *et al*, 2006b) we measured, on day 10 post infection the levels of these specific immunoglobulins as well as IgG_{2a} present in the intestinal mucosa and found no significant differences between high or low protein groups, although there was a tendency for high protein

groups to have increased antibody levels compared to the low protein groups. As well as humoral responses, we also measured the concentration of mucosal mast cells, rat mast cell protease (RMCP II), eosinophils, and aimed to measure globule leukocytes. Several studies have demonstrated that gastrointestinal nematode infections are invariably accompanied by these inflammatory cell responses (Miller, 1996), but we did not observe significant differences between treatment groups. This may have been due to the single sampling point used i.e. ten days post secondary infection. It might be argued that effects could be seen earlier or later than the time point used in our previous experiment. Connan (1973) found globule leukocytes from samples obtained after more than ten days of a single primary infection with *N. brasiliensis*, and it is known that a temporal relationship exists between elevated levels of IgE in serum and expulsion of parasites from the host (Olgivie & Jones, 1971: Miller & Jarrett 1971).

Therefore, the objective of the current experiment was to assess temporal effects of increased protein supply on resistance and immune responses to *N. brasiliensis* in lactating rats. It was hypothesised that the degree of *N. brasiliensis* infection occurring in the lactating rat would be sensitive to changes in protein scarcity at various time points during lactation. We expected that an increase in protein supply, achieved by feeding a high protein diet, on day 5 and day 15 post infection would result in reduced parasite burdens and affect local immune responses thought to be involved during expulsion of the nematode from the host.

5.3. Materials and Methods

5.3.1. Animals and housing

Forty eight second parity female Sprague-Dawley rats (Harlan Ltd, Oxfordshire, UK) were housed in a room where ambient temperature was maintained at 21°C, relative humidity ranged from 45 to 65%, and artificial lighting was provided between 07.00-19.00 hours. Rats were housed in solid-bottomed cages with fresh sawdust being provided weekly and a handful of shredded plastic bubble wrapping for nesting material from 3 days before expected parturition until the end of the experiment. Wire-bottomed cages were used during mating and for faeces collection during the primary infection as described previously (Houdijk *et al*. 2003a). For mating, female rats were placed with a proven male breeder and mating was confirmed through the presence of a vaginal plug.

5.3.2. Foods

All rats were given *ad libitum* access to standard rat chow until mating was confirmed. Mated rats were given *ad libitum* access to a high protein food (210g CP per kg DM) for 10 days followed by a low protein food (60g CP/kg DM) until parturition. This feeding protocol was used to reduce body protein reserves during the second half of gestation in order to maximize the degree of protein scarcity

during lactation when rats are on low protein foods (Pine *et al*. 1994; Houdijk *et al*. 2005a).

Parturition was considered as day 0 and from then rats were fed restrictively (7.5% of parturition body weight) one of two foods: low protein (LP), formulated to supply 100g CP/kg DM or a high protein (HP), formulated to supply 300g CP/kg DM. The level of restricted feeding used was based on the mean achieved dry matter intake and parturition body weight of the rats receiving the low protein diet from a previous experiment (Normanton *et al*, 2006a), so that the 7.5% level was expected to lead to the absence of refusals, and hence similar intake between treatment groups. The different crude protein levels of the two diets were achieved through the iso-energetic exchange of casein against a mixture of digestible carbohydrates and oil. Ingredients and chemical analysis of the experimental foods offered during lactation are shown in Table 1.

Table 1: Composition and analysis of diets used during lactation

Ingredients (g/kg fresh matter)	Experimental Diets	
	Low Protein, (LP)	High Protein, (HP)
Casein (plus 1% Methionine)	103	307
Starch	304	206
Sucrose	152	102
Corn oil	197	140
Vitamins	47	47
Minerals	47	47
Cornflour	46	46
Choline	7	7
Lecithin	2	2
Alphacel	94	94
Analysed chemical composition (g/kg dry matter)		
Dry matter (g/kg fresh)	772	659
Gross Energy (MJ/kg DM)*	19.7	19.7
Crude protein	120	323
Ether extract	171	148
Ash	49	41

* Calculated from feed tables

5.3.3. Infection protocol and experimental design

All rats were infected with *N. brasiliensis* according to a previously established protocol (Houdijk *et al*. 2003a). Rats received on day$_{-35}$ (35 days before the realised mean parturition date) a primary infection of 1,600 third-stage infective larvae L_3 of *N. brasiliensis*, which were suspended in 0.5 ml sterile phosphate buffered saline and given by subcutaneous injection above the hind leg. A secondary infection of 1,600 L_3 *N. brasiliensis* was administered on day 2 of lactation.

The experiment consisted of two treatment groups: high protein (HP) or low protein (LP), with sampling at; 5, 10 or 15 days post secondary infection. These corresponded to 7, 12, and 17 days into lactation. Each litter size was standardised at 9 pups by day 2 of lactation. The three time points during lactation were used to show the effects of differences in protein adequacy on the degree of parasitism and immune response over time, as the previous experiment (Normanton *et al*, 2006b) only sampled on day 10 post infection and failed to show any significant differences between high or low protein groups for immune responses.

The aim was to obtain 7 replicates for each of the six treatments. However, we started with 48 rats as it is known from previous experiments that ~ 10% would not conceive. Indeed, 5 rats did not conceive, but also 1 rat was euthanised, 1 rat died

during parturition, and 5 rats lost too many pups during lactation to provide viable results. Therefore, post hoc achieved number of replicates were $n = 7$ for LP5, $n = 6$ for LP10 and LP15, $n = 5$ for HP5 and HP10, and $n = 7$ for HP15.

The rats were killed on their allocated day for the assessment of colon egg count and immunological analysis, to assess the degree of parasitism. The standard protocol for worm burden collection does not allow for simultaneous collection of sufficient mucosal scrapings in order to measure immune responses.

5.3.4. Body weight and food intake

Rats were weighed daily throughout the experiment. Intake was measured daily during gestation and any refusals that did occur during lactation were collected every morning and weighed to measure achieved food intake. The pups were counted and weighed daily from day 0. Foods offered during gestation & lactation were sampled during their preparation, and were analysed for DM, crude protein (Kjeldahl-N x 6.25), ether extract and ash.

5.3.5. Nematode egg counts

Seven days post primary infection (day_{-30} relative to parturition) faeces were collected overnight as described previously (Houdijk *et al*. 2003a), for the assessment of faecal egg counts (eggs per g of faeces). This was done to provide evidence that a primary infection had established.

5.3.6. Nematode egg count during the secondary infection

All rats were sedated and humanely killed by CO_2 asphyxiation on day 5, 10 or 15 post secondary infection and dissected to collect the small and large intestine. Large intestinal contents were collected, weighed and assessed for the concentration of nematode eggs as described before (Houdijk *et al*. 2003a). A small 2 cm section of small intestine, 15 cm down from the stomach, was placed in 4% paraformaldehyde for 6 h and then transferred to 70% ethanol. The small intestine sections were then processed for the measurement of the concentration of mucosal mast cells, globule leukocytes, and eosinophils, as described previously (Huntley *et al*. 1995). Sections were also used for the staining of goblet cells. To confirm goblet cell numbers identified by morphology, they were localised using Alcian blue staining (Itoh et al, 1999). Positive staining (red) with Alcian blue indicates the presence of acid mucins, and negative staining (blue) with Alcian blue indicates the presence of neutral mucins within goblet cells.

5.3.7. *Mucosal scrapings*

Mucosa samples were obtained by scraping approximately 45cm of the anterior small intestine with a glass microscope slide. Samples were then transferred into a bijoux tube and frozen. For the analysis of antibody and rat mast cell protease (RMCP II), the samples were thawed and homogenised on ice, using ice-cold phosphate buffered saline (PBS), to solubilise the mucosa sample.

5.3.8. Analysis of mucosa samples for soluble antibody

An enzyme linked immunosorbent assay (ELISA) method was used for the detection of the antibody isotypes IgG_1, IgG_{2a}, IgA and IgE in mucosal tissue. High binding ELISA plates were coated with 10µg/ml of adult *N. brasiliensis* antigen, in 50 µl/well bicarbonate buffer pH9.6 for 1 hour. Plates were then washed three times with PBS/Tween 20/NaCl solution. Mucosal homogenates for each rat were used in dilution 1 in 10 in PBS. These were added in 50 µl/well PBS and incubated for 1 hour at room temperature, following which plates were washed six times as above. Isotype-specific secondary antibodies were then added (Serotec, UK). All secondary antibodies were diluted in PBS/Tween 80 at a 1/1000 dilution and incubated on the plates for 1h at room temperature. Following washing six times as above, horse radish peroxidase (HRP) conjugated anti-mouse immunoglobulin antibody (Serotec, UK) was added to each well (50µl). This was again incubated for 1 hour. Plates were then washed six times as above, and then the antibody isotypes could be detected by the addition of 50µl/well of Sigma Fast-OPD and the colour allowed to develop for around 15 minutes before stopping with 2.5 M H_2SO_4. Following colour development, optical density values (OD) for each well were read at 492 nm using a plate reader.

5.3.9. Analysis of mucosa samples for RMCP II

RMCP II concentration were detected and quantified from mucosa scrapings by ELISA as described by Miller et al (1983), using a commercially available kit (Moredun Scientific Limited, RMCP II ELISA). Assays were carried out according to manufacturer's instructions. Briefly, ELISA plates were coated with 2 µg/ml anti-RMCP II monoclonal antibody in bicarbonate buffer pH 9.6 at 4°C overnight. Following six washes with phosphate buffered saline with Tween-20 (PBST) plates were blocked for unspecific protein at 37°C for 30 min with 4% Bovine serum albumin in PBST. Plates were washed again three times and standards in series 0.5-12 ng/ml of RMCP II in PBST were added in. Mucosal homogenates were then plated in four dilution series of times ten, (1:10, 1:100, 1:1000, and 1:10,000) and incubated at 37°C for 30 min. Following a further six washes, plates were incubated for 1h with 50µl/well conjugated antibody solution at 37°C. Plates were once again washed six times before addition of 50 µl/well substrate (specific to the RMCP II kit) to allow for the colour formation. Colour formation was allowed to progress for around 15 min before stopping with 0.25M H_2SO_4. OD values for each well were then read at 450 nm on a microplate reader and RMCP II concentration calculated by comparison against a plotted standard curve.

5.3.10. Calculations and statistical analysis

Due to their skewed nature, colon egg count, inflammatory cell counts, antibody OD values and RMCP II concentrations were transformed according to log (n+1) to

normalize data before statistical analysis. The transformed data are reported as backtransformed means, accompanied by a backtransformed lower and upper limit. These were calculated via 10^{a}-1 where $a = \mu+0.5\times\sigma^2$ (Johnson et al., 1988), with μ, μ - s.e. and μ + s.e. as the mean, lower and upper limit of the transformed data, and σ as their standard deviation. Consequently, backtransformed lower and upper limits characterise an unequally distributed range around the backtransformed means, reflecting the skewed nature of the original data.

REML was used to: (1) test apriori the effect of assumed protein scarce state (LP9) vs assumed protein sufficient state (HP9) at each time point during lactation, and (2) to test post hoc the hypothesis that the secondary infection of *N. brasiliensis* was sensitive to an increased protein supply and time in lactating rats in using a 2x3 factorial design. The expectation was that parasitism would reduce and immune responses increase with an increased protein supply on day 5 or 15 post secondary infection. The 2 levels of feeding treatments were used to analyse the effects on nematode egg excretion, nematode eggs in colon, and immune responses (inflammatory cells, antibodies, and RMCP II).

The lactation period was divided into 3 groups in order to assess the effects of feeding treatments on dam body weight, dam feed intake, and litter body weight. Period 1 consisted of day 2-7 of lactation, period 2 was day 7-12, and period 3 was day 12-17, and the division into these periods was used to account for reducing replicates from the serial slaughter design. The effects of feeding treatments on dam body weight, dam feed intake, and litter body weight were assessed through repeated

measure ANOVA, taking into consideration the effect of time and interactions between feeding treatments and time. Body weight at parturition was used as a covariate when analysing the effects of treatments on maternal weight during lactation. All statistical analyses were performed using Genstat 7 for Windows (release 6.1, 2004; Lawes Agricultural Trust, Rothamsted, Herts., UK) and Minitab 12 (release 12.1, 1998; Minitab Inc.).

5.4. Results

5.4.1. Faecal egg counts during the primary infection and performance until parturition

All rats showed signs of a primary infection, and on day$_{-30}$ the mean faecal egg count reached 23,331 (18,424 – 29,545) eggs per gram.

During the first 10 days of gestation, rats grew from 265g (SE 5.9) to 297g (SE 7.2), with an average DM intake of 19.8 g/day (SE 0.3). From then onwards and until parturition, the pregnant rats continued to grow to a mean weight of 342g (SE 8.3) with an average DM intake of 14.3g/day (SE 0.2), which dropped to an average of 6.2g (SE 0.05) on the day before parturition.

5.4.2. Food intake, dam and litter body weight during lactation

Figure 1a shows the DM intake for the dams during lactation from day 2. During period 1 there was no significant differences for feed intake between treatment groups (P=0.04). During period 2, diet and time significantly interacted for DM intake (P=0.01). HP rats had significantly higher intakes than LP rats on day 8 (P=0.02), day 9 (P=0.01) and day 10 (P=0.01). Period 3 had no significant differences for feed intake between treatment groups (P=0.04).

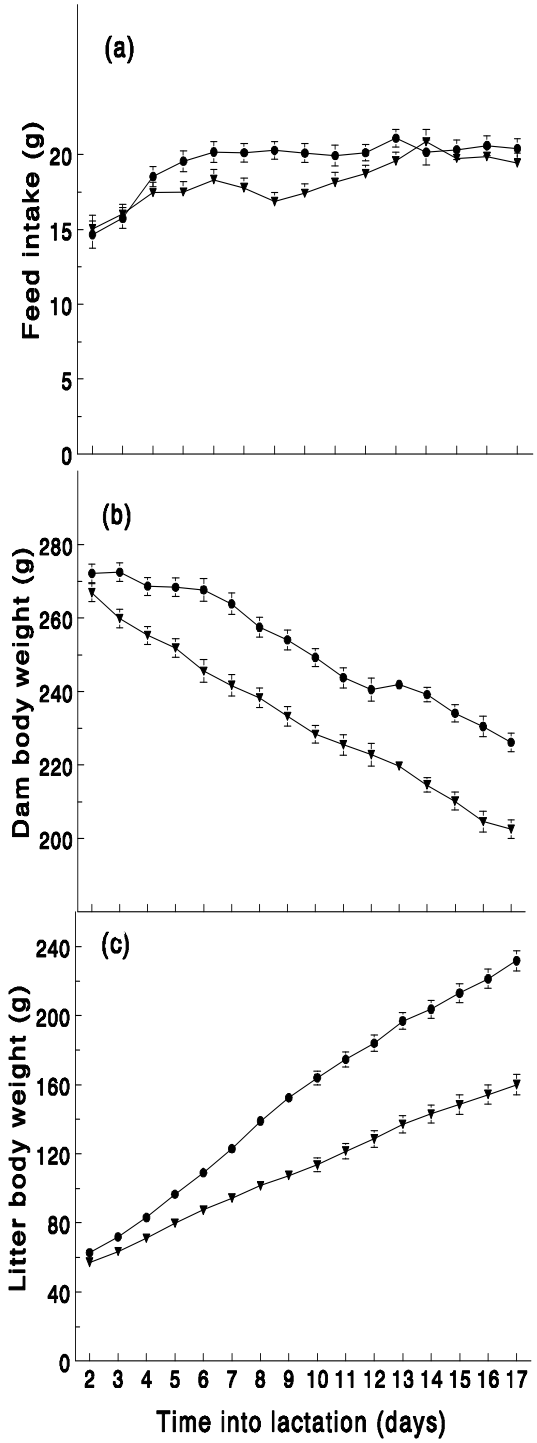

Figure 1: Dry matter dam food intake (with se) (a), least square mean (with se) dam body weight (b) and litter body weight (c) of lactating rats, restrictedly offered low protein foods (100g crude protein/kg DM) and nursing 9 (▲) pups, or high protein foods (300g crude protein/kg DM) and nursing 9 pups (●).

5.4.3. Dam performance

Figure 1b shows dam body weight during lactation from day 2. At parturition, mean dam body weight was 267 g (SE 5.2). During period 1 there was a trend for diet and time to interact for dam body weight (P=0.07). HP rats lost 0.5g whilst LP rats lost 22g (S.E.D 3.6g, P=0.001). Diet and time did not significantly interact for dam body weight during period 2 (S.E.D 22.6g, P=0.77) with HP rats losing 31g and LP rats losing 24g or during period 3 (S.E.D12.8g, P=0.74), with HP rats loosing 19g and LP rats loosing 18g. HP9 rats differed significantly from LP9 rats in final dam body weight at the end of lactation (P=0.002).

5.4.4. Litter performance

Mean litter weight from day 2 during lactation is shown in Figure 1c. At parturition, mean litter weight was 61 g (SE 5.4). During period 1, there was a trend for diet and time to interact (P=0.09). HP litter gain (44g) was higher than LP litter gain (37g) (S.E.D 3.0g, P=0.03). During period 2 diet and time significantly interacted for litter gain (P=0.05) HP litters gained 45g whilst LP litters gained 28g (S.E.D 15.0g, P=0.008). During period 3 diet and time significantly interacted for litter gain (P=0.03). HP litters gained 35g, whilst LP litters gained 23g (S.E.D 14.0g, P=0.001).

5.4.5.Colon egg count

The effect of diet on backtransformed mean number of eggs in the colon is shown in figure 2. Diet did not have a significant effect on day 5 (P=0.14). On day 10 HP rats had significantly fewer nematode eggs in their colon than LP rats (P=0.04), as on day 15 (P=0.07). Diet and time did not significantly interact for the mean number of eggs found in the colon (P=0.48). However, the main effects of diet and time were significant, with across time, HP rats having lower eggs than LP rats (P=0.002).

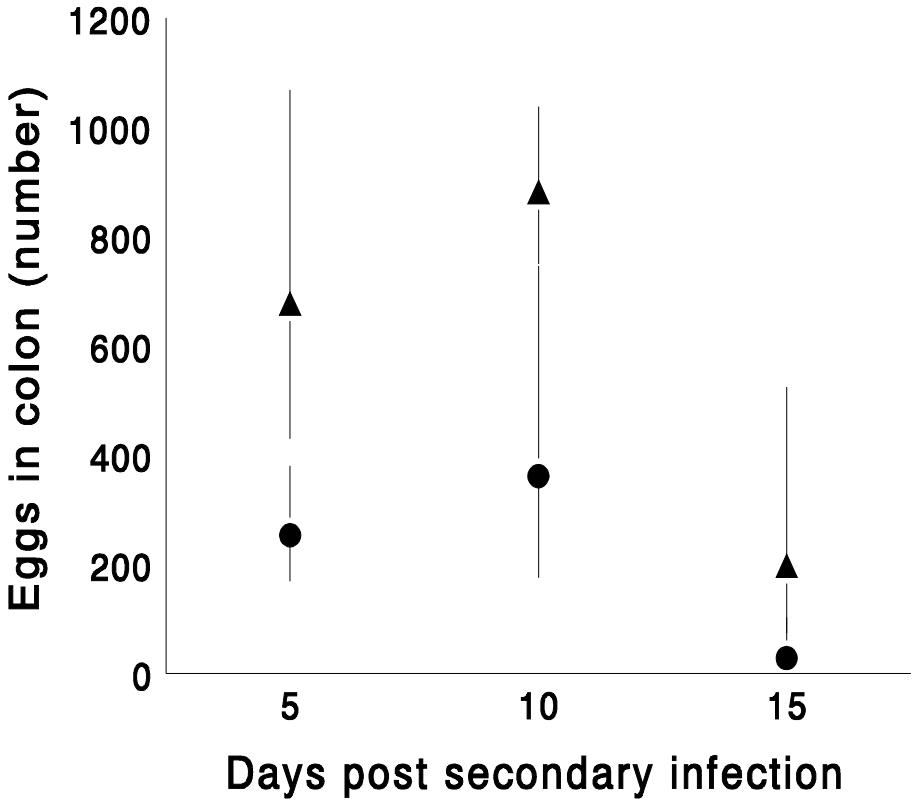

Figure 2. Mean backtransformed (with backtransformed standard errors) number of eggs in colon contents of lactating rats, restrictedly offered low protein foods (100g crude protein/kg DM) and nursing 9 (LP▲) pups on day 5, 10, and 15 post secondary infection or high protein foods (300g crude protein/kg DM) and nursing 9 pups (HP●).

5.4.6. Immunological analysis

As shown in figure 3, there was a trend for diet and time to interact for IgA (P=0.11), IgE (P=0.10) and IgG_{2a} (P=0.12) levels, with HP rats tending to have higher levels on day 5, compared to LP rats (P=0.07). There was no interaction between diet and time for IgG_1 levels (P=0.29). There was a trend for time (P=0.11) to affect the levels of immunoglobulin present in the mucosa. For HP rats their IgA levels gradually decreased from day 5 to day 15 (P=0.01), as did their IgE levels (P=0.05) and IgG*2a* levels (P=0.09). IgG_1 levels were not affected by time (P=0.24). Time did not significantly affect the level of immunoglobulin present in LP rats for IgA (P=0.87), IgE (P=0.71), IgG_1 (P=0.83) or IgG_{2a} (P=0.82).

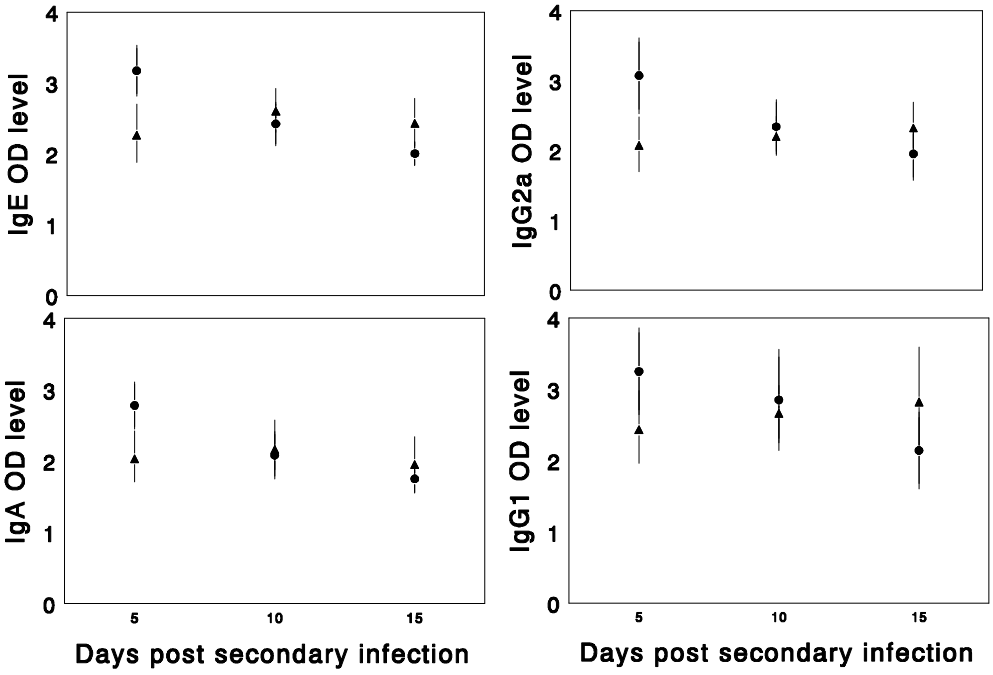

Figure 3. Mean backtransformed (with backtransformed standard errors) OD levels of IgA, IgE, IgG_1 and IgG_{2a} from lactating rats, restrictedly offered low protein foods (100g crude protein/kg DM) and nursing 9 (LP▲) pups on day 5, 10, and 15 post secondary infection or high protein foods (300g crude protein/kg DM) and nursing 9 pups (HP●).

5.4.7. Mucosal mast cells, eosinophils and RMCP II

Figure 4 shows the effect of diet on backtransformed number of mucosal mast cells, eosinophils, and concentration of RMCP II. Diet did not have a significant effect on day 5 (P=0.76), day 10 (P=0.51) or day 15 (P=0.39) on the concentration of mucosal mast cells found in the gut. There was no significant interaction between diet and time (P=0.69). The main effect of diet was not significant (P=0.14), but time was (P=0.001).

Diet had a significant effect on the concentration of eosinophils found in the gut, with HP rats have a much higher concentration day 5 (P=0.01) and day 15 (P=0.02). There was no significant effect on day 10 (P=0.33). Diet and time did not significantly interact (P=0.68). The main effect of diet was significant (P=0.001), but there was no main effect of time (P=0.51). Globule leukocytes were absent in all tested sections.

As shown in figure 4, diet did not significantly effect RMCP II levels on day 5 (P=0.37), however, on day 10 (P=0.003) and day 15 (P=0.002) HP rats had significantly lower concentrations of RMCP II than LP rats. Diet and time did not significantly interact (P=0.18), with diet having a significant main effect on RMCP II levels (P=0.001), but there was no significant main effect of time (P=0.66).

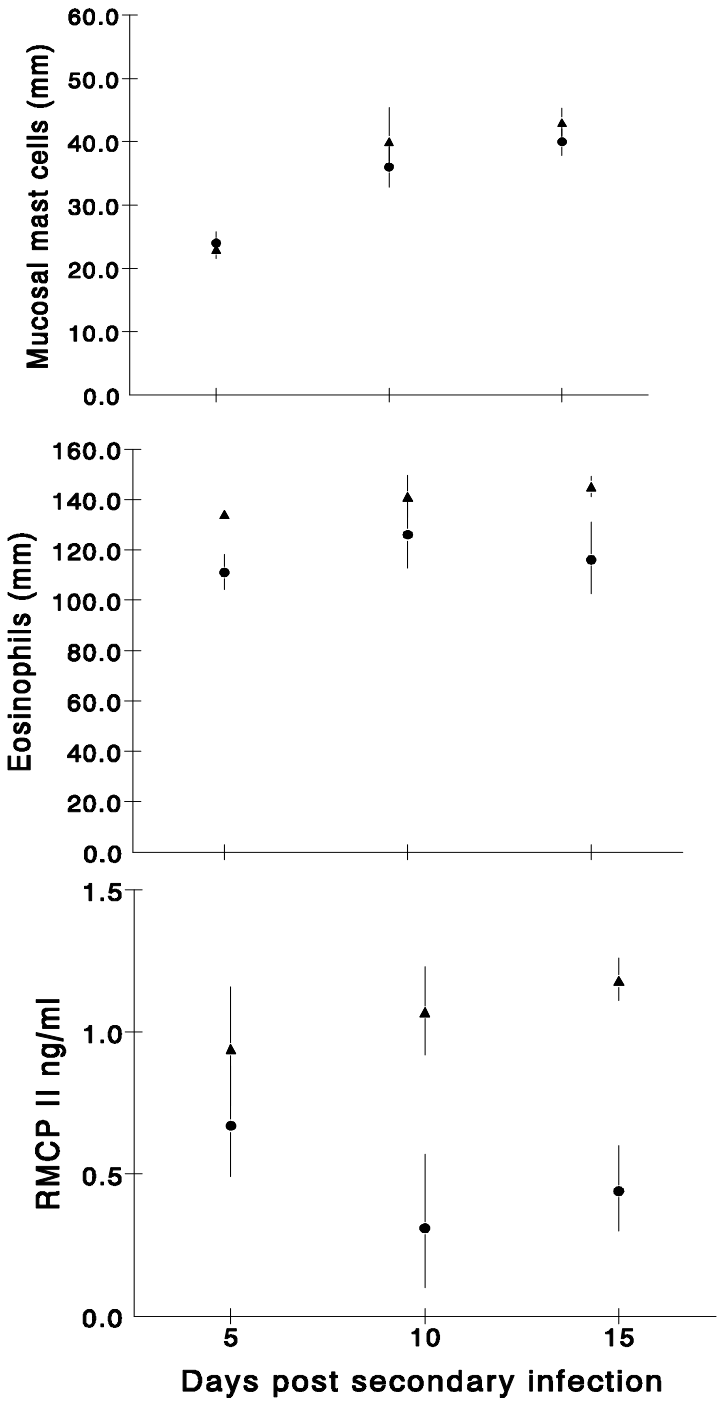

Figure 4. Mean backtransformed (with backtransformed standard errors) RMCP II concentrations, mucosal mast cell numbers and eosinophil numbers from lactating rats, restrictedly offered low protein foods (100g crude protein/kg DM) and nursing 9 (LP▲) pups on day 5, 10, and 15 post secondary infection or high protein foods (300g crude protein/kg DM) and nursing 9 pups (HP●).

5.4.8. Goblet cells

Figure 5 shows the effect of diet on backtransformed total number of goblet cells, neutral mucin cells and acidic mucin cells present in the intestinal mucosa. Diet had a significant effect on day 5. HP rats had significantly more acidic cells (P=0.01), neutral cells (P=0.09) and total number of goblet cells (P=0.007) compared to LP rats. On day 10, HP rats had significantly more acidic cells (P=0.02) than LP rats, but no significant differences were seen for neutral (P=0.73) or total number of goblet cells (P=0.15). Diet did not have a significant effect on day 15 for acidic cells (P=0.95), neutral cells (P=0.16), or total number of goblet cells (P=0.51). There was a trend for diet and time to interact for the number of acidic mucin cells (P=0.11) but no such interaction occurred for neutral mucin cells (P=0.51), or total number of goblet cells (P=0.52). Diet had a significant main effect (P=0.009), whereas time was not significant (P=0.52).

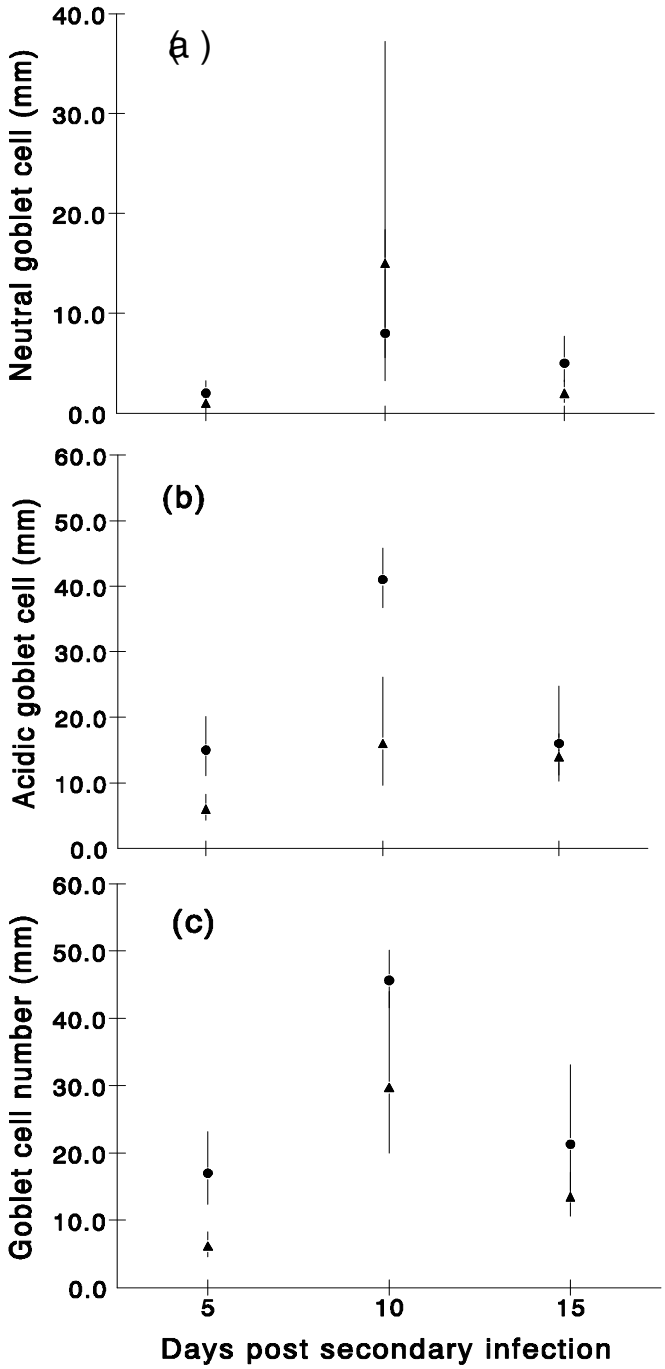

Figure 5. Mean backtransformed (with backtransformed standard errors) acid (a), neutral (b), and total (c) goblet cell numbers from lactating rats, restrictedly offered low protein foods (100g crude protein/kg DM) and nursing 9 (LP▲) pups on day 5, 10, and 15 post secondary infection or high protein foods (300g crude protein/kg DM) and nursing 9 pups (HP●).

5.5. Discussion

It has been shown that lactating rats infected with the gastrointestinal nematode *N. brasiliensis*, can significantly reduce their parasitic burden through either an increase in protein supply or by a decrease in nutrient demand (Houdijk *et al*., 2005, Normanton *et al*, 2006a). Although these experiments were able to show that differences in nutrient scarcity significantly affected the level of parasitism, they were unable to demonstrate nutritional sensitivity of underlying immune responses associated with the expulsion of the nematode. A possible explanation may be that there was only a single sampling point on day 10 post infection. The current experiment aimed to account for this uncertainty by testing the hypothesis that parasitism during lactation is sensitive to protein supply and that this sensitivity may be associated with temporal effects on immune responses to *N. brasiliensis*.

The effects of changes in protein supply were to be evidenced by comparing HP and LP feeding treatments. From the data obtained, it is evident that these feeding treatments affected dam performance i.e., body weight change and litter gain (proxy for milk production). Although there was no significant difference between HP and LP dam body weight loss during period 2 or 3, HP rats lost less body weight compared with LP rats during period 1, causing final HP dam body weight to be significantly higher than LP dam body weight (fig 1b). HP litter gain was also significantly higher than LP litter gain during all 3 time periods (Fig 1c). These results therefore suggest that protein scarcity was less in HP rats than in LP rats, and that the correct conditions had been created in order for the hypothesis to be tested.

The results from the present study show that differences in protein supply affected the number of nematode eggs present in the colon. HP rats had lower number of eggs compared to LP rats, especially on day 10 and 15. In order for the diets to have the same gross energy content, it was necessary to iso-energetically exchange casein for carbohydrates/oil, which resulted in the diets slightly differing in their metabolisable energy content. However, this was less than 3%, and it has been shown that expression of immunity to gastrointestinal parasites is not sensitive to such moderate changes in energy supply (Bown *et al*, 1991, Donaldson *et al*, 1998). As well as differing slightly in metabolisable energy content, the LP diet also contained more fat than the HP diet. However, effects of fat intake on the immune system are usually related to the intake of n-3 rather than n-6 fatty acids (Yaqoob, 2004) and the latter would be the case from the maize oil used in the present experiment. Therefore, we suggest that the differences seen in the number of eggs present in the colon between the LP and HP rats was likely due to an increased intake of protein.

It is believed that adult *N. brasiliensis* are expelled from the small intestine by a two step mechanism. Step 1 comprises of antibodies causing irreversible damage to the parasite. This is followed by step 2 comprising of the actual expulsive process of non-specific inflammatory responses induced by the "damaged" worms (Ishiwata *et al*, 2002). Once the parasites are cleared from the primary infection the animal retains an effective immunological memory, transferable by immune cells (Kelly & Dineen 1972). This memory manifests itself by a more rapid expulsion in

subsequent infections commencing around day 7 of re-infection (Olgilve & Love 1974). Cummins et al, (1987) found that protein deficiency reduced the number of mucosal mast cells (MMC) compared to normal fed rats on day 5 and 15 post infection. For this reason, our current experiment aimed to assess effects of protein supply on immune responses on day 5, 10 and 15 post secondary infection.

Primary intestinal nematode infections are typically accompanied by elevated IgE, IgA and IgG1 antibody isotypes (Onah & Nawa, 2000, Negrao-Correa *et al*, 1999). However, there is little evidence to indicate whether these antibodies are involved in worm expulsion in lactating animals experiencing a breakdown of immunity. Our results showed that there was a trend for HP rats to have higher levels of IgA, IgE and IgG_{2a} on day 5 post infection. As effects were seen on day 5 post infection, and worm expulsion may peak on day 7 during a secondary infection, these results suggest that the antibodies role may indeed have been to "damage" the worm, before other non-specific inflammatory responses initiate worm expulsion. This is supported by an absence of differences in nematode egg number in the colon on day 5 post infection. Increased protein supply had temporal effects on IgA, IgE, and IgG_2a levels which caused them to gradually decrease from day 5 to day 15, which were reflected in the number of eggs present in the colon. However no such effects were seen for the LP rats. It could be speculated that over time, HP rats were able to expel more worms and/or reduce their fecundity even before expulsion, due to the increased level of IgA present on day 5. As LP rats had lower levels of IgA on day 5 compared to HP rats, they had a higher number of worms throughout lactation which would indicate that their immunoglobulin levels would not be able to gradually

decrease overtime as they still need to effectively damage and then expel the worm. It has been shown in previous experiments using lambs that the magnitude of the IgA response is influenced by increased protein nutrition (Strain & Stear, 2001). This has also been seen in mice infected with *Trichinella*, where studies have suggested a strong temporal correlation between mucosal IgA production and a reduction in fecundity and size of the adult worm, but not the rate of expulsion, in challenge infections (Robinson *et al*, 1995).

Th2 type responses associated with gastrointestinal nematode infections also includes specific inflammatory cells. There is uncertainty regarding the degree of nutritional sensitivity of the mast cell response to *N. brasiliensis* infection. Cummins *et al* (1987) showed that dietary protein deficiency in growing rats delayed expulsion of *N. brasiliensis,* which concurred with a reduction in the number of mucosal mast cells compared to protein-replete controls. However, more recent studies have suggested that the final phase of immune expulsion of *N. brasiliensis* in primary infections is mast cell independent (Nawa *et al*, 1994). The current results are in agreement with this view, as although our HP rats had increased worm expulsion compared to LP rats, as indicated by differences in colon egg count, protein supply had no significant effect on the number of mucosal mast cells present in the small intestine. However, these results do not mean that MMC do not play an important role in the elimination process of the nematode. It could be speculated that during an infection, similar numbers of MMC are recruited to the gastrointestinal tract for HP and LP rats, but they vary in their release of rat mast cell protease (RMCP II). RMCP II is a mediator which is localised exclusively in MMC (Gibson & Miller

1986), and it has been reported that increased mucosal permeability is associated with expulsion of a primary *N. brasiliensis* infection, which coincides with maximal release of RMCP II (Nawa *et al*, 1979). The results from the current experiment show that LP rats had significantly higher levels of RMCP II on day 10 and 15 post secondary infection compared to HP rats. It is noticeable that RMCP II may have direct damaging effects on the nematode (Hyoh *et al*, 1999). LP rats had significantly higher levels on day 10 and 15 as these rats still had high levels of worms present in the small intestine. The LP treatment hinders the final immune response which would normally expel the worms. Therefore, high levels of RMCP II may be expressed to cause damage to the worms present in the gut, even though final expulsion is not possible due to a later immune response, which is believed to be goblet cell dependent (Nawa *et al*, 1994).

Our analysis showed that increasing protein supply had a significant effect on the number of eosinophils present in the gastrointestinal tract on day 5 and day 15 post secondary infection. These observations are in contrast with results from our previous work, where a reduction in nutrient demand and protein supply was associated with a reduction in worm burden, but no significant differences were seen for the level of eosinophils on day 10 post secondary infection (Normanton *et al*, 2007; Normanton *et al,* 2006). These results would indicate that eosinophils might play a role in the lead up to final worm expulsion, although the temporal effects observed are not consistent. However, given that the lung is a possible site of parasite attrition in immune animals (Ferens *et al*, 1990) it is also possible that they have an earlier role. Eosinophils could affect migratory larvae in transit from the

skin to the stomach via the tissues and lungs (Shin *et al*, 1997), which could be investigated in future experiments.

After appropriate antigenic stimulation, mucosal mast cells release their contents and gradually develop into transitional cells and then into globule leukocytes (Huntley, 1992). The current experiment aimed to also analyse the number of globule leukocytes present in the small intestine. However, globule leukocytes were absent in all of the mucosal tissues collected. This is in contrast to previous findings where the presence of an effect of protein supplementation on globule leukocytes has been consistently observed in other parasitised mammals (Houdijk *et al*. 2005b). However, in these studies a gradual, trickle infection has been used, in comparison to our short, single period of infection. This might explain why globule leukocytes were absent in the mucosal samples. There is a chronological association between goblet cell hyperplasia and worm expulsion in primary *N. brasiliensis* infections (Nawa *et al*, 1994). However, no such studies have looked at the role of goblet cells during worm expulsion of a secondary infection in lactating rats receiving different protein supplies. Our results have shown that the effect of increased protein supply causes an increase in the total number of goblet cells on day 5 post secondary infection. These results are in agreement with a studied carried out by Cummins et al, (1987), where a reduced goblet cell response and mucus changes in protein-deficient rats receiving a primary *N. brasiliensis* infection indicated an impaired immune response. Also, earlier experiments have indicated that chronic protein depletion decreases goblet cell number and mucin synthesis in rodent and pig small intestine (Neutra *et al*, 1974; Sherman *et al,* 1985). As well as measuring the total

number of cells, we also stained for qualitative changes in goblet cell mucins and found that HP rats had significantly higher acidic mucins on day 5 and 10 post infection. No differences were seen between neutral mucins for HP and LP rats during lactation. Immature goblet cells produce neutral incomplete mucins (Isselbacher, 1974), and the observed increase in the number of acid mucin containing goblet cells from day 5 post secondary infection are due to mature sulfo-mucin containing goblet cells (Forstner *et al*, 1982). Therefore, this study, together with previous observations, not only supports the view that quantitative/qualitative changes of goblet cell mucins play a role in the expulsion of *N. brasiliensis*, but also provides first evidence that goblet cell hyperplasia is sensitive to an increase in protein supply during lactational breakdown of immunity.

In conclusion, the results of this experiment support the view that the extent of *N. brasiliensis* infection during the periparturient period is sensitive to changes in protein supply. The effect of increased protein supply on immune responses has provided novel evidence that IgA, IgE, IgG_{2a}, RMCP II and goblet cells are some of the underlying mechanisms which are nutritionally sensitive to the relaxation in immunity during the periparturient period.

General Discussion

6.1 Introduction

This thesis has provided novel information on the nutritional sensitivity of immune responses associated with gastrointestinal nematode infections during a breakdown of immunity. The hypothesis that has been developed to account for the effects of increasing scarce nutrient supply on the periparturient relaxation in immunity is based on a nutrient-partitioning framework. This framework proposes that the relaxation in immunity occurs due to an increased nutrient requirement of the prioritised reproductive effort at times when nutrient supply is scarce (Coop & Kyriazakis, 1999). Therefore, during late pregnancy and lactation, if the periparturient animal receives a secondary nematode infection, she will be unable to mount an effective immune response due to her reproductive effort taking priority over her immune response. Scarce nutrients will be allocated primarily to her reproductive effort.

To test for the effects of increasing scarce nutrient availability on the degree of breakdown of immunity in the lactating animal, an already established rodent model was used. Four separate experiments were carried out in chronological order. The first experiment (Chapter Two) aimed to test the effect of changing nutrient (litter) demand whilst nutrient supply was maintained constant. This would verify that a reduction in worm burden is indeed related to changes in nutrient supply. A subsequent experiment (Chapter Three) broadened this approach and investigated the effect of increased protein supply or reduced protein demand on the resistance to parasites in lactating rats whilst energy intake was kept constant. Under these

conditions effects of protein supply could not be confounded with effects of any nutrient or energy intake. Following on from this, the third experiment (Chapter Four) assessed the effects of a gradual increase in protein supply on resistance and immune responses to *N. brasiliensis* in lactating rats. This experiment was needed as it was not known how gradual increments of scarce metabolisable protein would affect milk production and the breakdown of immunity. Finally, the last experiment (Chapter Five) assessed the effects of increased protein supply on resistance and immune responses to *N. brasiliensis* at various time points during lactation, as it was thought that immune responses may occur earlier or later than the single sample time point used in the previous experiments.

The principal issues raised by the experimental studies, which warrant further discussion in this chapter are:

- The effects of changing nutrient supply or demand on the degree of *N. brasiliensis* infection.
- The effects of scarce metabolisable protein on the degree of immunity breakdown.
- Effects of scarce metabolisable protein on associated immune responses.
- The efficacy of the rodent model. The potential application of using a lactating rat as a suitable model to fully understand the underlying immunological basis of relaxation in immunity during the periparturient period

Subsequently, special reference is given to future research lines to further investigate the nutritional sensitivity of the periparturient animals' immune responses during a breakdown of immunity.

6.2 The rodent model and its sensitivity to protein

Although there have been many experiments investigating the immune responses associated with the rat and the gastrointestinal nematode *N. brasiliensis* during nematode expulsion (Rothwell, 1998; Onah & Nawa 2001; Nawa *et al*, 1994, Maizels *et al*, 1994; Miller 1996) there have been few investigations into the nutritional sensitivity of the immune responses (Connan, 1972; Cummins 1987). These experiments have only focused on the effect of nutrient supply on the immune response during a primary infection. However, more recently there has been renewed interest in creating a rodent model to investigate the effects of altering nutrient supply during a breakdown of immunity as alternative, drug free parasite controls are being sought. Houdijk *et al* (2003a, 2005a) created a rodent model to assess whether a reduction in nutrient scarcity during lactation resulted in a reduced degree of parasitism. Feeding high protein foods resulted in a reduced worm burden, but was confounded with increased food intake *per se*. Therefore, effects observed on parasitism may not necessarily have been associated with an increased nutrient supply, but could have been related to changes in the gut environment or increases in energy intake, as a consequence of increased food intake. This raised the important question of whether it was actually the scarce protein supply affecting the degree of breakdown in immunity. The aim of the experiments shown in Chapter Two and

Three was to validate the nutritional sensitivity of the model towards scarce metabolisable protein supply. The results shown in Chapter two demonstrated the effects of changes in nutrient demand on the degree of breakdown of immunity. Rats fed a low protein diet during lactation had a clear reduction in worm burden as their litter size was reduced, although there were no significant differences between feed intake. This indicated improvement of host resistance in the absence of direct changes in gut environment. In addition, the results from chapter three aimed to illustrate the effect of increasing scarce protein supply on the number of eggs present in the colon during a breakdown of immunity. Lactating rats with a similar food intake, during the post infection period, had a clear reduction in the number of eggs in the colon with increased protein supply and reduced nutrient demand. At similar feed intake, high protein rats with a litter size of nine pups had a significantly lower number of eggs compared to low protein rats with a litter size of nine pups. Therefore, these first two experiments helped to develop and validate the model created by Houdijk *et al* (2003a, 2005a), and support the view that the reduction in the degree of breakdown of immunity is likely achieved through effects of protein availability on expression of immunity, and not through changes in the gastrointestinal environment.

Subsequently, the aim of chapter 4 was to focus on the effects of gradually increasing protein supply on lactational performance and immunity to *N. brasiliensis*. Previous experiments have used only one level of scarce and one of adequate protein nutrition, and consequently, they provide only limited insight in the relationship between protein supply and immunity to parasites, and no insight in the degree of penalty of

protein scarcity on lactational performance and resistance to *N. brasiliensis*. Based on the nutritional framework proposed by Coop & Kyriazakis (1999), it was hypothesised that scarce metabolisable protein will be firstly allocated to lactational performance, and only when the rat reaches a protein abundant state will there be an improved immunity, evidence by a reduction in nematode egg count and worm burden. However, our results showed that in contrast to expectation, worm burden and the number of nematode eggs present in the colon concurrently reduced with increased litter gain, suggesting increased milk production. This would imply that scarce metabolisable protein is partitioned to milk production as well as expression of immunity. A study carried out by Houdijk *et al*, (2003*b*) also observed a similar relationship between metabolisable protein supply, milk production and nematode egg excretion, but not final worm burden in sheep. However, due to decreased food intake of the high protein rats and a low number of replicates, as explained in chapter four, this experiment would need to be repeated again in order to be able to reach a final conclusion on the effect of gradually increasing protein supply on the priority of allocation to reproduction effort and immune responses.

6.3 The effect of protein supply on associated immune responses

There have been many laboratory models of intestinal nematode infections which have played an important role in developing our understanding of the immune mechanisms that operate against infectious agents. These have mostly used primary infections and have shown that there is a close association between intestinal nematode infection and the generation of mastocytosis, eosinophilia,

immunoglobulin production and secretion of goblet cell mucus (Maizels *et al*, 2004). However, it has been consistently difficult to be able to demonstrate a role for these effector cells in resistance to nematodes (Else & Finkelman, 1998), and there have been no studies investigating the effect of nutrition on the local immune responses during a breakdown of immunity. Therefore, the main aim of experiment four (Chapter Five) was to investigate the effects of metabolisable protein on the local immune responses associated with a secondary infection *of N. brasiliensis* on day 5, 10 or 15 post secondary infection. Although experiments one, two and three aimed to show how differences in MP availability actually affected local immune responses, we only sampled on day 10 and the obtained results failed to show any significant differences.

The results shown in chapter five have provided novel evidence on the effect of increased protein supply on immune responses during a breakdown of immunity. Firstly, they have shown that mucosal mast cells may indeed play a role in the resistance to an infection, which is in contrast from the results shown in experiments one, two and three. In these earlier studies it was found that there were no differences in the number of mucosal mast present in the small intestine, even though it was evident that an increase in MP availability caused a significant reduction in the degree of *N. brasiliensis* infection. These results were in agreement with several studies where it was shown that mast cells were not required to successfully expel a *N. brasiliensis* infection (Madden et al, 1991, Crowle & Reed, 1981, Urban et al, 1998). However, a more recent theory is that mast cells may contribute to a non-specific inflammatory response within the gut through the secretion of inflammatory

mediators such as RMCP II and leukotrienes (Scudamore et al, 1995). The results from experiment four (Chapter Five) have shown that although there are no significant differences between the number of mast cells present in the gut, on days 10 and 15 post secondary infection rats receiving a high protein diet had low worm burdens and lower levels of RMCP II compared to rats receiving a low protein diet with high worm burdens.

As well as RMCP II, we have found that the responses of IgA, IgE and IgG2a during a breakdown of immunity towards *N. brasiliensis* are also sensitive to changes in MP availability. Increasing scarce MP during lactation resulted in higher levels of IgA, IgE and IgG_{2a} on day 5 post secondary infection, but not on day 10 or 15. As effects were seen on day 5 post infection, and worm expulsion may peak on day 6-7 during a secondary infection, these results suggest that the antibodies role may indeed have been to "damage" the worm, before other non-specific inflammatory responses initiate worm expulsion. This is supported by an absence of differences in nematode egg number in the colon on day 5 post infection. These results provide valuable information as relatively little attention has been paid to local antibody production as used in our study, as opposed to peripheral. It is believed that antibodies secreted at mucosal surfaces may yet prove to be important in protective immunity to gut dwelling helminths (Else & Finkelman, 1998), and our results are in agreement with this belief.

The final immune responses shown to be sensitive to changes in MP availability are goblet cells and their mucins. It is well known that that during a primary infection

many intestinal nematode infections induce a goblet cell hyperplasia and an increase in mucin production (Ishikawa *et al*, 1993). However, the role of goblet cell in protective immune response and whether it is sensitive to changes in nutrient supply has not received much attention. Our results (Chapter Five) have shown that this study, together with previous observations, not only supports the view that quantitative/qualitative changes of goblet cell mucins may play a role in the expulsion of *N. brasiliensis*, but also provides first evidence that goblet cell hyperplasia is sensitive to an increase in protein supply during lactational breakdown of immunity. Rats receiving the high protein diet had a reduced worm burden and increased number of goblet cells compared to rats receiving a low protein diet. These results are in agreement with a studied carried out by Cummins et al, (1987), where a reduced goblet cell response and mucus changes in protein-deficient rats receiving a primary *N. brasiliensis* infection indicated an impaired immune response. Also, earlier experiments have indicated that chronic protein depletion decreases goblet cell number and mucin synthesis in rodent and pig small intestine (Neutra *et al*, 1974; Sherman *et al,* 1985).

From these results we have been able to show that IgA, IgE, IgG_{2a}, RMCP II and goblet cells are some of the underlying mechanisms which are nutritionally sensitive to the relaxation in immunity during the periparturient period.

6.4 Efficacy of our rodent model

The aim of the thesis was to develop a rodent model to be able to assess the underlying nutritional sensitivity of immune responses associated with a breakdown of immunity. We have shown that increasing the metabolisable protein availability to a periparturient animal suffering from a breakdown of immunity reduces the number of eggs present in the colon, reduces the degree of worm burden and affects the underlying mechanisms associated with the expulsion of *N. brasilienis*. The results on the effect of metabolisable protein availability through changes in supply or demand, on the parasitic burden are highly comparable to results seen in other periparturient animals experiencing a breakdown of immunity. For example, Kahn et al, (2003) showed that postpartum protein supplementation reduced faecal egg count of periparturient ewes by 66% compared to non supplemented ewes. These results are consistent with a number of other studies (e.g. Houdijk *et al*. 2001, 2003b, 2005; Donaldson *et al*. 1998, 2001) which have all shown that an increase in protein during lactation helps to reduce worm burden. It has also been shown that growing animals also experience a relaxation in immunity and benefit from an increased protein supply during this period. Parasitised growing lambs receiving increased dietary protein diets had decreased faecal worm egg counts and increased live weight gain (Datta et al, 1998). In addition to showing effects of protein supply, these studies, as well as other ones have shown the effects of changes in protein demand. Parasitised single-rearing ewes have usually lower faecal egg counts and smaller worm burdens than their twin-rearing counterparts (Kahn et al, 2003, Houdijk *et al* 2001;

Donaldson *et al*. 1998), whilst similar effects have been seen in low-yielding dairy goats compared to high producing goats (Chartier *et al*. 2000).

These results all compare favourably with our results, hence, the evidence obtained to date shows that our *N. brasiliensis* lactating rat model can reproduce similar nutritional sensitivities of host resistance to gastrointestinal nematode parasites during the periparturient period as observed in other lactating hosts. This supports the view that this model may be used to assess nutritional sensitivity of periparturient immune responses that may be associated with nutritionally improved resistance to parasites.

It is known that there are differences in individual immune responses and effector mechanisms associated with worm expulsion dependent on the species of helminth. For example, there is compelling evidence that the mast cell plays a major role in the resistance to an infection of *T. spiralis*, but it is known that mast cells are not directly responsible for the expulsion of a *N. brasiliensis* infection (Tuohy *et al,* 1990). It is also known that effector responses to *T. spiralis* following infection are different in the mouse compared to the rat (Robinson *et al*, 1995). However, in general, resistance to intestinal nematode infections correlates with the ability to mount a Th2 type immune response. In order to expel an intestinal nematode infection, CD4 + T cells are required to make Th2 type cytokines, and each species of nematode requires a unique set of Th2 type cytokines to initiate expulsion (Else & Finkelman, 1998). Our rodent model has shown that during a breakdown of immunity to *N. brasiliensis*, typical Th2 type immune responses have occurred such as mastocytosis, goblet cell

hyperplasia, immunoglobulin production, and that these responses are more than likely sensitive to changes in nutrient supply. These results are in agreement with other studies which have used different rodent models to look at the effect of dietary protein supply on immune responses during nematode infections. Boulay *et al* (1998) found that mice given a secondary infection of *Heligmosomides polygyrus* and fed low protein diet had significantly higher worm burdens and egg output compared to mice fed a high protein diet. Ing *et al* (2000) also found that protein malnutrition increased the survival of *H. polygyrus*, and they concluded this was associated with a decrease in gut-associated IL-4 and increased IFN-gamma, which leads to reduced intestinal and systemic Th2 effector responses. In addition to these experiments, Gbakima (2006) showed that mice primarily infected with *Trichinella spiralis* and given a low protein diet had delayed and weakened inflammatory responses to the invading parasites compared to normal protein diets. The similar results seen in these experiments as well as in our rodent model have also been shown in studies using periparturient sheep or growing lambs. Increased dietary protein has resulted in increased IgA levels (Strain & Stear, 2001), increased levels of plasma IgE (Houdijk *et al,* 2005). However, whereas Ing *et al* (2000) found a depressed mucosal mast cell proliferation in low protein mice compared to high protein mice, our results are more in agreement with Houdijk *et al* (2005) where it was shown that differences in protein supply during lactation had no effect on the number of mucosal mast cells found in the small intestine of ewes (Houdijk *et al*, 2005).

In conclusion, our rodent model has proven itself to work reliably and produce results which are in agreement with various other studies, and can therefore be used as a viable model to further investigate the nutritional sensitivity of the immune responses associated with worm resistance/expulsion during a breakdown of immunity.

6.5 Future directions

This thesis has provided novel evidence on the effect of host nutrition on immune responses associated with the expulsion of *N. brasiliensis* during a breakdown of immunity. Thus, the model can now be used to further investigate the full extent of nutritional effects on the breakdown of immunity during lactation or growth. This will provide a fully researched alternative for anthelmintics to aid parasitoligical control in farming animals.

As changes in immune responses can be very small, as seen in our findings, we would recommend for future work to increase the number of animals per treatment group to improve the experimental power. Taking this into consideration, the immunological results could be further expanded to include the various type 2 cytokines known to be important in the regulation of antiparasite immune responses, such as IL-4, IL-3 and IL-5 (Finkelman *et al*, 1997).

In addition, the results from chapter five could be the basis for further investigating the effects of metabolisable protein supply on immune responses at earlier time points during lactation, or even on every lactation day to gain a more detailed insight. It would also be of interest to investigate the effects of individual amino acids on the associated immune responses, to see whether specific ones contribute to the enhancement of the immune responses.

Finally, it would be possible to use this rodent model to investigate the effects of nutrition on the molecules and pathways that underlie the protective or pathologic consequences of the type 2 inflammation during a breakdown of immunity. The focus could be on four families of proteins that are highly induced in helminth infections: 1) the arginases; 2) the resistin-like molecules; 3) the chitinase-like mammalian proteins; and 4) the intelectins (Nair, Guild & Artis 2006).

Bibliography

Abe T & Nawa Y (1988) Worm expulsion and mucosal mast cell response induced by repetitive IL-3 administration in *Strongyloides ratti* infected nude mice. *Immunol.* **63**, 181-185.

Agricultural and Food Research Council (1993) Energy and protein requirements of ruminants. An advisory manual prepared by the AFRC Technical Committee on responses to Nutrients, CAB International, Wallingford.

Arizono N, Kasugai T, Yamada M, et al. (1993) Infection of *Nippostrongylus brasiliensis* induces development of mucosal-type but not connective tissue-type mast cells in genetically mast cell deficient Ws/Ws rats. *Blood* **81**, 2572-2578.

Athanasiadou S, Kyriazakis I, Jackson F & Coop RL (2001) The effects of condensed tannins supplementation of foods with different protein content on parasitism, food intake and performance of sheep infected with *Trichostrongylus colubriformis*. *Br J Nutr* **86**, 697-706.

Barger IA (1993) Influence of sex and reproductive status on susceptibility of ruminants to nematode parasites. *Int. J. Parasitol.* **23**, 463-469.

Befus AD & Bienenstock J (1979) Immunologically mediated intestinal mastocytosis in *Nippostrongylus brasiliensis* infected rats. *Immunol* **38**, 95-101.

Befus AD (1995) Immune responses: protective immunity, adaptation and pathogenesis. In: Enteric Infection 2 (MJG Farthing, GT Keusch, D Wakelin, eds), Chapman and hall Medical, London, 1st ed. Pp. 49-70.

Berghen P, Dorny P, Hilderson H, Vercruysse J & Hollanders W (1990) Observations on parasitic gastroenteritis and parasitic bronchitis in calves over two grazing seasons. *Vet. Rec.* **127**, 426-430.

Boulay M, Scott ME, Conly SL, Stevenson MM & Koski KG (1998) Dietary protein and zinc restrictions independently modify a *Heligmosomoides polygyrus* infection in mice. *Parasitol.* **116**, 449-462.

Bown MD, Poppi DP, Sykes AR (1991) The effect of post-ruminal infusion of protein or energy on the pathophysiology of *Trichostrongylus colubriformis* infection and body composition in lambs. *Austr J Agric. Res.* **42**, 253-267.

Capron M, Capron A, Torpier G, Bazin H, Bout D & Joseph M (1978) Eosinophil-dependent cytoxicity in rat schistosomiasis. Involvement of IgG_{2a} antibody and role of mast cells. *Eur J. Immun.* **8**, 127-133.

Chandra RK (1989) Nutritional regulation of immunity and risk in infection in old age. *Immunology* **67**, 141-147.

Chartier C, Etter E, Hoste H, Pors I, Mallereau M-P, Broqua C, Mallet S, Koch C & Masse A (2000) Effects of the initial level of milk production and of the dietary protein intake on the course of natural nematode infection in dairy goats. *Vet. Parasitol* **92**, 1-13.

Clunies-Ross, I. & Graham, N.P. (1932). Parasitological field trials with sheep. Results at "Gundowringa", New South Wales, and "Frodsley" Tasmania. *Journal for the Council for Scientific and Industrial Research* **5**:31-39.

Connan RM (1973) The immune response of the lactating rat to *Nippostrongylus brasiliensis*. *Immunology* **25**, 261-267.

Connan RM (1970) The effect of host lactation on the self-cure of *Nippostrongylus brasiliensis in rats*. *Parasitol.* **61**, 27-33.

Coop RL & Holmes PH (1996) Nutrition and parasite interaction. *Int. J. Parasitol.* **26**, 951-962.

Coop RL & Kyriazakis I (2001) Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. *Trends Parasitol* **17**, 325-330.

Coop RL, & Kyriazakis I (1999) Nutrition-parasite interaction. *Vet. Parasitol* **84**, 187-204.

Coop RL, Mellor DJ, Jackson E, Jackson F, Flint DJ & Vernon RG (1990) *Teladorsagia circumcincta* egg output at the onset of natural and induced lactation in ewes. *Vet. Parasitol.* **35**, 295-305.

Crowle & Reed (1981) Rejection of the intestinal parasite *Nippostrongylus brasiliensis* by mast-deficient W/Wv anaemic mice. *Infect. Immunity*, **33 (1)**, 54-58.

Cummins AG, Duncombe VM, Bolin TD, Davis AE & Young J (1987) The response of the small intestine of the protein-deficient rat to infection with *Nippostrongylus brasiliensis*. *Int. J. Parasitol* **17**, 1445-1450.

Datta FU, Nolan JV, Rowe JB & Gray GD (1998) Protein supplementation improves the performance of parasitised sheep fed a straw-based diet. *Int. J. Parasitol* **28**, 1269-1278.

Dent LA, Daly CM, Mayrhofer G, Zimmerman T, Hallett A, Bignold LP, Greaney J and Parsons JC (1999) Interleukin-5 transgenic mice show enhanced resistance to primary infections with *Nippostrongylus brasiliensis* but not primary infections with *Toxocara canis*. *Infect. Immunity* **67**, 989-993.

Dinnen JK & Kelly JD (1972) The suppression of Rejection of *Nippostrongylus brasiliensis* in Lactating Rats: the Nature of the Immunological Defect. *Immunol* **22**, 1-12.

Donaldson J, van Houtert MFJ & Sykes AR (1998) The effect of nutrition on the periparturient parasite status of mature ewes. *Anim. Sci.* **67**, 523-533.

Donaldson J, van Houtert MFJ & Sykes AR (2001) The effect of dietary fish-meal supplementation on parasite burdens of periparturient sheep. *Anim. Sci.* **72**, 149-158.

Else KJ, Finkelman FD (1998) Intestinal nematode parasites, cytokines and effector mechanisms. *Int. J. Parasitol.* **28**: 1145-1158.

Ferens WA, Arai HP & Befus AD (1990) Trickle infections with *Nippostrongylus brasiliensis* in rats: larval migration through lungs. *J. Parasitol* **76**, 684-689.

Finkelman FD, Shea-Donohue T, Goldhill J, Sullivan CA, Morris SC, Madden CB, Gause WC & Urban JF (1997) Cytokine regulation of host defense against parasitic gastrointestinal nematodes. *Annu. Rev. Immunol* **15**, 505-533.

Forstner G, Wesley A & Forstner J (1982) Clinical aspects of gastrointestinal mucus. In Mucus in Health and Disease II, 199-224.

Friggens NC, Hay DEF & Oldham JD (1993) Interactions between major nutrients in the diet and the lactational performance of rats. *Br J Nutr* **69**, 59-71.

Gbakima AA (2006) The effect of dietary protein on *Trichinella spiralis* infection and inflammatory reactions in the tingue in CD1 mice. *Nut. Res*. **13**, 787-800.

Giacomin PR, Wang H, Gordon DL, Botto M & Dent LA (2005) Loss of complement activation and leucocyte adherence as *Nippostrongylus brasiliensis* develops within the murine host. *Infec. Immun.* **73**, 7442-7449.

Gibson S & Miller HRP (1986) Mast cell subsets in the rat distinguished immunohistochemically by their content of serine proteinases. *Immunol.* **58**, 101-104.

Gray GD (1997) The use of genetically resistant sheep to control nematode parasitism. *Vet. Parasitol.* **72**, 345-366.

Haley J (1962) Biology of the rat nematode *Nippostrongylus brasiliensis*. *J Parasitol.* **48**, 13-23.

Hammond JA, Fielding D & Bishop SC (1997) Prospects for plant anthelmintics in tropical veterinary medicine. *Vet. Res. Comm.* **21**, 213-228.

Herndon F & Kayes SG (1992) Depletion of eosinophils by anti-IL-5 monoclonal antibody treatment of mice infected with *Trichinela spiralis* does not alter parasite burden or immunological resistance to re-infection. *J. Immunol.* **149**, 3642-3647.

Houdijk JGM, Jackson F, Coop RL & Kyriazakis I (2006) Rapid improvement of immunity to *Teladorsagia circumcincta* is achieved through a reduction in the demand for protein in lactating ewes. *Int J Parasitol* **36**, 219-227.

Houdijk JGM, Jessop NS, Knox DP, & Kyriazakis I (2005a) Secondary infection of *Nippostrongylus brasiliensis* in lactating rats is sensitive to dietary protein content. *Br. J. Nut.* **93**, 493-499.

Houdijk JGM, Kyriazakis I, Jackson F, Huntley JF & Coop RL, (2005b). Effects of protein supply and reproductive status on local and systemic immune responses to *Teladorsagia circumcincta* in sheep. *Vet. Parasitol* **129**, 105-117.

Houdijk JGM, Jessop NS, Knox DP, & Kyriazakis I (2003a). Breakdown of immunity to *Nippostrongylus brasiliensis* in lactating rats. *Br. J. Nut* **90**, 809-814.

Houdijk JGM, Kyriazakis I, Jackson F, Huntley JF & Coop RL (2003b). Is the allocation of metabolisable protein prioritised to milk production rather than to immune functions in *Teladorsagia circumcincta*-infected lactating ewes? *Int. J. Parasitol **33**, 327-338.*

Houdijk JGM, Kyriazakis I, Jackson F & Coop RL (2001a). The relationship between protein nutrition, reproductive effort and breakdown in immunity to *Teladorsagia circumcincta* in periparturient ewes. *Anim. Sci.* **72**, 595-606.

Houdijk JGM, Jessop NS & Kyriazakis I (2001b) Nutrient partitioning between reproductive and immune functions in animals. *Pr. Nut. Soc.* **60** (4): 515-525.

Houdijk JGM & Athanasiadou S (2003) Direct and indirect effects of host nutrition on ruminant gastrointestinal nematodes. *VI Int. Sym. Nutr Herb.*

Hudson RJ & White RG (1985) Bioenergetics of wild herbivores. Publisher: Boca Raton, FLA: CRC Press.

Houtert, M.F.J., & Sykes, A.R. (1996). Implications of Nutrition for the Ability of Ruminants to Withstand Gastrointestinal Nematode Infections. *Int. J. Parasitol* **26**:1151-1168.

Huntley JF (1992) Mast cells and basophils: a review of their heterogeneity and function. *J. Comp Path.* **107**, 349-372.

Huntley JF, Patterson, Mackellar A, Jackson F, Stevenson LM & Coop RL (1995) A comparison of the mast cell and eosinophil responses of sheep and goats to gastrointestinal nematode infections. *Res Vet Sci* **58**, 5-10.

Hyoh Y, Nishida M, Yamada M, Uchikawa R, Matsuda S & Arizono N (1999) Enhancement of apoptosis with loss of cellular adherence in the villus epithelium of the small intestine after infection with the nematode *Nippostrongylus brasiliensis* in rats. *Parasitol.* **119**, 199-207.

Ing R, Su Z, Scott ME & Koski KG (2000) Suppressed T helper 2 immunity and prolonged survival of a nematode parasite in protein malnourished mice. *Proc. Nation. Acad. Sci.* **13**, 7078-7083.

Ishikawa N, Horii Y, Oinuma T, Suganuma T & Nawa Y (1994) Goblet cell mucins as the selective barrier for intestinal helminths: T-cell independent alteration of goblet cell mucins by immunologically "damaged" *Nippostrongylus brasiliensis* worms and its significance on the challenge infection with homologous and heterologous parasites. *Immunol.* **81**, 480-486.

Ishiwata K, Nakao H, Nakamura-Uchiyama F & Nawa Y (2002). Immune mediated damage is not essential for the expulsion of *Nippostrongylus brasiliensis* adult worms from the small intestine of mice. *J. Immunol.* **24**, 493-499.

Isselbach KJ (1974) Intestinal cell surface- some properties of normal, undifferentiated, and malignant cells. *Ann. Intern. Med.* **81**, (5) 681-686.

Itoh H, Beck PL, Inoue N, Xavier R & Podolsky DK (1999) A paradoxical reduction in susceptibility to colonic injury upon targeted transgenic ablation of goblet cells. *J. Clin. Invest.* **104**, 1539-1547.

Jarrett EEE, Jarrett WFH & Urquhart GM (1968) Quantitative studies on the kinetics of establishment and expulsion of intestinal nematode populations in susceptible and immune hosts. *Nippostrongylus brasiliensis* in the rat. *Parasitol.* **58**, 625-639.

Jessop, N.S. (1997). Protein metabolism during lactation. *Proceedings of the Nutrition Society* **56**:169-175.

Johnson N.L., Kotz S., and Balakrishnan N. 1998. Continuous Univariate Distributions. Vol. 1, 2nd. PWS-KENT, Boston.

Kahn LP (2003) Regulation of the resistance and resilience of periparturient ewes to infection with gastrointestinal nematode parasites by dietary supplementation. *Aus J Exp Agr* **43**, 1477-1485.

Katona, IM, Urban, JF & Finkelman, FD (1988) The role of L3T4+ and Lyt-2+ T cells in the IgE response and immunity to *Nippostrongylus brasiliensis*. *Parasite Immunol* **140**, 3206-3211.

Kelly JD & Dineen JK (1972) The suppression of rejection of *Nippostrongylus brasiliensis* in the rat by promethazine hydrochloride. The site of immunological impairment. *Immunol.* **22**, 361-370.

Koski KG & Scott ME (2001) Gastrointestinal nematodes, nutrition and immunity: breaking the negative spiral. *Annu. Rev. Nutr.* **21**, 297-321.

MacRae JC (1993) Metabolic consequences of intestinal parasitism. *Proc. Nutr. Soc.* **52**, 121-130.

Lawrence CE (2003) Is there a common mechanism of gastrointestinal nematode expulsion? *Parasite Immunol*. **25**, 271-281.

Lawrence RA, Gray CA, Osborne J & Maizels RM (1996) *Nippostrongylus brasiliensis*: cytokines responses and nematode expulsion in normal and IL-4 deficient mice. *Exp. Parasitol*. **84**, 65-73.

Lochmiller RL, Vestey MR & McMurry ST (1993) Selected immune responses of adult cotton rats to dietary restriction. *Comp. Biochem. And Physiol.* **104**, 593-599.

Madden KB, Urban JF, Ziltener HJ, Schrader JW, Finkelman FD & Katona IM (1991) Antibodies to IL-3 and IL-4 suppress helminth induced intestinal mastocytosis. *J Immunol* **147**, 1387-1391.

Maizels RM, Balic C, Gomez-Escobar N, Nair M, Taylor MD, & Allen JE (2004). Helminth parasites-masters of regulation. *Immun. Rev*. **201**, 89-116.

Maizels RM & Holland MJ (1994) Parasite immunity: Pathways for expelling intestinal helminths. *Cur Biol* **8**, R711-R714.

McKenzie GJ, Bancroft A, Grencis RK, McKenzie NJ (1998) A distinct role for interleukin-13 in Th2-cell-mediated immune responses. *Cur Biol* **8**, 339-342.

Miller HRP & Jarrett WFH (1971). Immune reactions in mucous membranes. I. Intestinal mast cell response during helminth expulsion in the rat. *Immunol,* **20**, 277-288.

Miller HRP, Woodbury RG, Huntley JF, (1983) Systemic release of mucosal mast-cell protease in primed rats challenged with *Nippostrongylus brasiliensis*. *Immunol* **49**, 471-479.

Miller HRP (1996) Mucosal mast cells and the allergic response against nematode parasites. *Vet Immunol Immunopathol* **54**: 331-336.

Mosmann TR & Coffman RL (1989) Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol*. **7**, 145-173.

Nair GM, Guild KJ & Artis D (2006) Novel effector molecules in type 2 inflammation: Lessons drawn from helminth infection and allergy. *J Immunol*.

National Research Council (1995) Nutrient Requirements of Laboratory Animals, 4th ed. Washington, DC: National Academy Press.

Nawa Y (1979) Increased permeability of gut mucosa in rats infected with *Nippostrongylus brasiliensis*. *Int. J. Parasitol*. **9**, 251-256.

Nawa Y, Ishikawa N, Tsuchiya K, Horii Y, Abe T, Khan AL, Bing-shi Itoh H, Ide H & Uchiyama F (1994) Selective effector mechanisms for the expulsion of intestinal helminths. *Parasite Immunol* **16**, 333-338.

Negrao-Correa D, Adams LS, & Bell RG (1999) Variability of the intestinal immunoglobulin E response of rats to infection with *Trichinella spiralis*, *Heligmosomoides polygyrus* or *Nippostrongylus brasiliensis*. *Parasite Immunology* **21,** 287-297.

Neutra MR, Maner JH & Mayoral LG (1974) Effects of protein calorie malnutrition on the jejunal mucosa of tetracycline treated pigs. *Am J Clin Nutr* **27**, 287-295.

Newlands GF, Miller HR, MacKellar A, Galli SJ (1995) Stem call factor contributes to intestinal mucosal mast cell hyperplasia in rats infected with *Nippostrongylus brasiliensis* or *Trichinella spiralis*, but anti-stem cell factor treatment decreases parasite egg production during *N. brasiliensis* infection. *Blood* **86**, 1968-1976.

Nieuwhof GJ & Bishop SC (2005) Costs of the major endemic diseases of sheep in Great Britian and the potential benefits of reduction in disease impact. *Anim. Sc* **81**, 23-29.

Normanton H, Houdijk JGM, Knox D & Kyriazakis I (2007) The effects of changes in nutritional demand on gastrointestinal parasitism in lactating rats. *Br. J. Nut.* **97**, 104-110.

Normanton H, Houdijk JGM, Knox D & Kyriazakis I (2006) The effects of changes in protein supply and demand on gastrointestinal parasitism in lactating rats at a constant level of energy intake. *Proceedings of British Society of Animal Science* p 107.

Ogilvie BM & Jones VE (1971) *Nippostrongylus brasiliensis*; Review of immunity and the host-parasite relationship in the rat. *Exp. Parasitol* **29**, 138-177.

Ogilvie BM & Love RJ (1974) Cooperation between antibodies and cells in immunity to a nematode parasite. *Trans. Rev*. **19**, 147-168.

Onah DN & Nawa Y (2000) Mucosal immunity against parasitic gastrointestinal nematodes. *The Korean Journal of Parasitology* **38**, No.4, 209-236.

O'Sullivan BM & Donald AD (1970) A field study of nematode parasite populations in the lactating ewe. *Parasitol.* **61**, 301-315.

Petkevicus S, Nansen P, Bach KKE & Skjoth F (1999) The effect of increasing levels of insoluble dietary fibre on the establishment and persistence of *Oesophagostomum dentatum* in pigs. *Parasite* **6**:17-26.

Pine AP, Jessop NS & Oldham JD (1994) Maternal protein reserves and their influence on lactational performance in rats. *Br. J. Nut* **71**, 13-27.

Robinson K, Bellbaby T & Wakelin D (1995) Immunity to *Trichinella spiralis* transferred by serum from vaccinated mice not protected by immunization. *Par Immunol* **17**, 85-90.

Rothwell TLW (1988) Immune expulsion of parasitic nematodes from the alimentary tract. *Int. J. Parasitol*. **19**, 139-168.

Scudamore CL, Thornton EM & Mcmillan L (1995) Release of mucosal mast cell granule chymase, rat mast cell protease II, during anaphylaxis is associated with rapid development of paracellular permeability to macromolecules in rat jejunum. *J. Exp. Med.* **182 (6)**, 1871-1881.

Sherman P, Forstner J, Roomi N, Khatri I & Forstner G (1985) Mucin depletion in the intestine of malnourished rats. *Am J Physiol Gastrointest Liver Physiol* **248**, 418-423.

Shin EH, Osada Y, Chai JY, Matsumoto N, Takatsu K and Kojima S (1997) Protective roles of eosinophils in *Nippostrongylus brasiliensis* infection. *Int. arch. Allerg. Appl. Immunol*. **144**:45-50.

Strain SAJ & Stear MJ (2001) The influence of protein supplementation on the immune response to *Haemonchus contortus*. *Para. Immunol*. **23** (10), 527-531.

Sykes AR (1994) Parasitism and production in farm animals. *Anim. Prod.* **59**, 155-172.

Thamsborg SM, Roepstorff A & Larsen M (1999) Integrated and biological control of parasites in organic and conventional production systems. *Vet. Parasitol*. **84**, 169-186.

Tuohy M, Lammas DA & Wakelin D (1990) Functional correlations between mucosal mast cell activity and immunity to *Trichinella spiralis* in high and low responder mice. *Para. Immunol*. **12** (6), 675-685.

Urban JF, Noben-Trauth Dn Donalson DD *et al*. (1998) IL-13, IL-4Ralpha, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite *Nippostrongylus brasiliensis*. *Immunity* **8**, 255-264.

Wells PD & McHugh M (1983) Effect of dietary zeolite on host and parasite in rats infected with the nematode *Nippostrongylus brasiliensis*. *Zeolites*, **3**,353-359.

Yaqoob P (2004) Fatty acids and the immune system:from basic science to clinical applications. *Proc Nutr Soc* **63**, 89-104.